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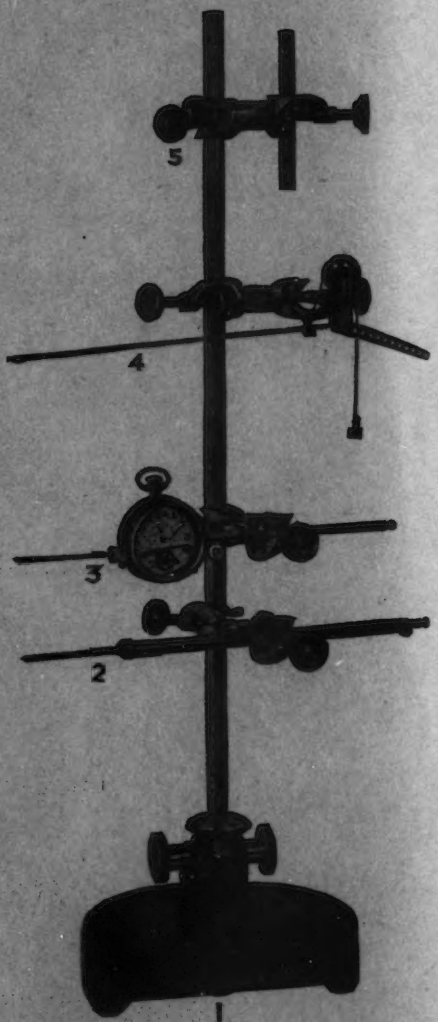

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DYE-METHODS FOR DETERMINING THE BLOOD-VOLUME TESTED IN VITRO

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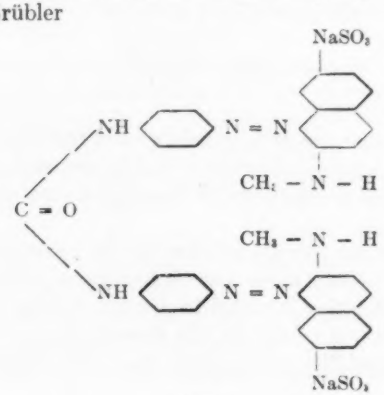
Keith, Rowntree and Geraghty (1915) in their original paper on this subject devised a method which was tested in vitro on diluted blood and proved to be reliable. As, however, dilution of the blood circulating in the living subject is impossible, this control cannot be accepted as sufficient. It will be shown below that undiluted blood may behave in a quite different manner.

The extensive papers on the dye-methods issued from the California University (1920–21) comprise only two control experiments in vitro; in these experiments the dye-solution was added to the undiluted blood, but afterwards the dye-colored as well as the uncolored plasma was strongly diluted. Such a control is also insufficient, in spite of the apparently satisfying results. To clear up the question, especially whether the dye-substance injected in the vessels or mixed with the shed blood is taken up only by the plasma-colloids or, perhaps, to a certain extent adheres to the red corpuscles, a more extensive and more close examination of the methods concerned is needed.

I. EXPERIMENTS ON OX-BLOOD. The experiments to be given in this section were carried out on oxalated ox-blood from the public slaughterhouse. The dye-substance used was in the majority of the experiments vital red from Grübler



in a watery 1 per cent solution; in some few experiments Congo-red (the sodium-salt of benzidindiazobinaphthylaminsulphonic acid) was used. The amount of dye-solution was varied, but in most cases 0.3 cc. of the solution was added to some 200 cc. of blood. After thoroughly mixing 2×1 cc. of the colored blood were placed in narrow glass-tubes (length about 6 cm. outer diameter 6 mm., 1 cc. divided in twentieths) and centrifuged at nearly 3000 revolutions per minute for one hour and a quarter. Then the hematocrit-reading proved to be equal to a reading obtained by means of an ordinary capillary hematocrit-tube when centrifuged to constant volume and translucency. Simultaneously two samples of the original blood (1 or 2 cc. each) were centrifuged in order to procure uncolored plasma necessary for comparison. The dimensions of the glass-tubes and the amount of blood were chosen with regard to the clinical application of the method. Reading of the four tubes gave the erythrocyte-volume within 1 per cent.

The colorimetry was in most of the experiments carried out according to the routine indicated by S. P. L. Sörensen (1909). A series of standard solutions of the dye in distilled water were made; 1 cc. of each were put into a tube of the dimensions given above and placed before a similar tube containing uncolored plasma; before the tube containing the colored plasma was placed a tube filled with distilled water. Now the tubes were changed until the color of the dye-plasma was found to lie between two of the adjacent tubes on the scale of standards. In the best part of the color-scale the difference in concentration between two neighboring tubes is 0.02 per cent, and thus the concentration of the dye in the colored plasma can be determined with certainty within these limits. From the known dye-concentrations the plasma-volume may easily be calculated. Thus if 0.3 cc. of the dye-solution is added to a sample of blood and the color of the dye-plasma is found to range between the scale-tubes containing 0.16 and 0.18 per cent of the dye-solution we have:

$$\text{Plasma-volume} = \frac{0.3 \times 100}{0.17} = 177 \text{ cc.}$$

The plasma-volume can with certainty be determined within the limits 167 to 187 or with a maximum error of $\pm$ 5.65 per cent but in the hands of an experienced observer the error of the reading may easily be reduced to the half.

Of course errors are introduced also by the measuring off of the dye-solutions. Yet it is only when a small fraction of a cubic centimeter of a concentrated dye-solution is added to a small portion of blood that such errors may be felt. An error in the dye-volume of 0.005 cc. which is already beyond possibility causes, when the routine followed in the experi-

ments on ox-blood here described is adopted, and when the pipette is washed out by means of blood from the sample, an error on the plasma determination of about ± 2 per cent. Regarding the preparations of the standard dye-solutions the same source of error is at play. When the color scale is used the relative agreement of a color tube with the neighboring tubes furnish a valuable control against the more serious errors. Also the reading of the blood-volume in the measuring glass may involve an error when the blood after shaking is foaming; an error of this kind never exceeds ± 1 cc. however. The hematocrit which has been controlled by means of an ordinary capillary hematocrit-tube may be read off to 1 per cent.

Now all the irregularities named have nearly the same absolute magnitude whether the blood-sample is large or small, and the possible error expressed in per cent of the blood-volume is, therefore, much larger when examining small blood-samples than when big samples are used.

As however the accuracy of the method especially the colorimetry depends on the experience of the observer it would be desirable to have a method more easy to manage. We therefore in a number of experiments compared the results obtained as described above with readings on a Bürker's colorimeter.

In the colorimeter the colored plasma as well as the uncolored "control"-plasma is enclosed in a glass-cell exactly 10 mm. in height; beneath these cells two open glass-vessels are placed containing distilled water and the standard dye-solution respectively. By dipping down two cylindrical glass-rods in these vessels, the thickness of the layer of dye-solution is varied until the color of the two fields of vision is exactly similar. The thickness of the layer is read with an accuracy of one-tenth of a millimeter on the scale of the apparatus, and from this by means of a very simple calculation the concentration of the dye-substance in the colored plasma is obtained. The final calculation of the plasma-volume is made in the manner described above. The reading of the colorimeter is most easily and accurately carried out in artificial light, especially, when the light is filtered through a solution having exactly the complementary color of the colored plasma. Then the field of vision appears greyish, and we obtain a very marked turning point of the variable part of the field. The colorimeter may be read off with an accuracy ranging from ± 0.3 to ± 0.1 mm. for the inexperienced and the experienced observer respectively. Regarding the readings given in the table below the accuracy may be taken as ± 0.2 mm. which involves an uncertainty on the determined plasma-volume of 2 to 4 per cent.

Comparable results of readings of the color-scale, respectively colorimeter, in 5 experiments are given in the table.

Thus the two methods give consistent results, and we resolved to make

use of the colorimeter for the volume-determinations in man as described in a succeeding paper.

The principal advantages offered by the colorimeter are: 1. It permits, especially to the unexperienced observer, a more accurate reading. 2. Only 1 cc. of plasma is needed to fill the glass-cell. 3. The readings are taken in artificial light. 4. Only one standard solution is needed. This is a rather important point, as the diluted dye-solutions only keep constant for a very limited space of time. 5. Minor differences in the shade of color which render the reading on the color-scale difficult vanish in the colorimeter when a suitable filter is used. On the other hand, any turbidity of the liquids examined which may perhaps be tolerated when the color-scale is used renders the colorimeter reading very inaccurate or impossible.

It has been pointed out repeatedly by previous writers that hemolysis makes colorimetry impossible; this is true, indeed, if the hemolysis has reached an appreciably high level, but if it is only slight there are no

TABLE 1

PLASMA-VOLUME, CC.		DIFFERENCE	
Color-scale	Colorimeter	In cubic centimeters	In per cent
188	200	+12	6.4
193	190	−3	1.6
256	257	+1	0.4
278	263	−15	5.4
308	303	−5	1.6

technical difficulties. Colorimetry is rendered impossible if the shade of color is altered to any appreciable extent or if the translucency of plasma is diminished as, e.g., in lipemia, but a small amount of hemoglobin has no influence in this regard. On the other hand even a slight degree of hemolysis makes the result very fallacious; that is to say, the results are—for quite unknown reasons—some 10 per cent higher, than is the case when no hemolysis has taken place. Hemolysed blood cannot, therefore, be used for blood-volume determinations by means of the methods here in question.

As already referred to, it is a necessary condition for an exact reading of the colorimeter to have—at least very nearly—the same shade of color in the solutions to be compared, only the intensity of color may vary. When using the vital-red mentioned above this condition is always fulfilled, even if a very slight degree of hemolysis is present. If on the other hand the above named Congo-red is added to the blood we always find a different shade of color, when comparing colored plasma with uncolored plasma + dye-solution. If control-plasma is not added to the standard dye-solution, the color-difference is of course far more pronounced and

makes in fact the comparison quite impossible. When as well the shade as the intensity of color are varied simultaneously, the readings become uncertain to such a degree that every value desired may be obtained. Even artificial yellow light as recommended by Bennhold (1923) does not secure a reliable reading. Therefore, Congo-red cannot be used for blood-volume determinations.

If we always make use of control-plasma, dye-solution may be added repeatedly to the same portion of blood, and the experiment as well as the calculation may be carried out in the usual manner.

The following table comprises 11 experiments performed with all due precautions on oxalated ox-blood; the adequate amount of powdered oxalate being added. No hemolysis could be detected by spectroscopical

TABLE 2

DATE	BLOOD-VOLUME	VITAL-RED	HEMATOCRIT	PLASMA VOLUME		
				Calculated	Determined	Error on the determination
	cc.	*cc.*	*per cent*			*per cent*
22/9	246	0.5	50	123	182	48.0
3/10	196	0.3	44	110	154	40.0
3/10	200	0.3	43	114	158	38.5
13/9	204	0.3	41	120	162	35.0
7/11	395	0.6	41	233	308	32.0
5/11	310	0.5	40	186	256	37.5
6/11	340	0.5	40	204	278	36.0
1/10	210	0.3	37	132	171	29.5
1/10	160	0.24	36	102	130	27.5
25/9	200	0.3	35	130	172	32.0
17/9	202	0.3	35	131	167	27.5

examination of the plasma. The table is arranged by decreasing values of the hematocrit-reading.

It is seen that the method applied to ox-blood involves a very serious error. Generally an error of this order of magnitude would render the method useless. Since, however, in the present case the error is fairly proportional to the volume of the red cells a correction might possibly be applied to the result. The relation between the cell-volume and the errors of the determinations is shown in figure 1. In this and the following figures the circles indicate the possible error on the colorimeter reading; other errors are not considered.

The proportionality between the error on the plasma-determination and the hematocrit-reading indicates that the error is due to absorption of dye-substance by the red cells, and this assumption is supported by other facts. Thus the error decreases when the blood is diluted, being zero, when the

erythrocyte-volume is reduced to about 10 per cent of the total blood. It seems, however, that the dilution proper has some influence on the behavior of the dye-substance in the blood; at least the percentage error on the determination, when working with diluted blood, does not follow exactly the straight line found in the above cited experiments on undiluted blood but takes a more complex course as shown in table 3 and figure 2. We do not, however, propose to pursue this problem further, because the determinations of the blood-volume in man must be carried out on undiluted blood. As mentioned above, hemoglobin set free by hemolysis seems to have a special attraction to the dye, making the determined amount of plasma too high, i.e., higher than is found when applying the same method to the same blood before hemolysis has taken place; thus if we make a series of successive determinations on blood in which hemolysis is going on, the error on the plasma-volume increases from day to day. These facts might indicate that hemoglobin possesses a special affinity to the dye, the

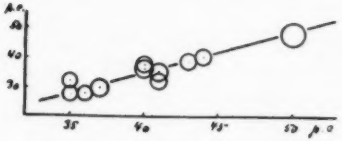


Fig. 1

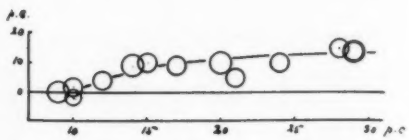


Fig. 2

Fig. 1. Experiments on undiluted ox-blood. Abscissa: Volume of the red cells. Ordinate: Error on the plasma determination.

Fig. 2. Experiments on diluted ox-blood. Abscissa: Volume of the red cells. Ordinate: Error on the plasma determination.

constitution of which is altered. On the other hand it is evident that the dye in some way or other is taken up by the stromata of the red cells, as can be demonstrated directly if we, after centrifugalizing the colored blood and removing the plasma, hemolyse the red corpuscles by means of distilled water and a small quantity of ether. When now the stromata are thrown down by centrifugalizing, and the residuum thus obtained is washed 3 to 4 times by distilled water it will appear colored, while the residuum from uncolored blood treated in exactly the same manner will appear uncolored greyish-white. The idea that dye is adsorbed to the stromata is not opposed by the fact that dilution of the blood influences the error on the plasma-volume caused by the red cells in a more complicated manner than is explained by the simple decrease in number. It is reasonable to assume that the adsorption is governed partly by, e.g., the viscosity of the fluid in which the red corpuscles are suspended, and it is evident that the physical properties of the solution is altered by dilution. A measure of the effect of dilution we have in the change in the rate of precipitation of the

red cells which was increased in all the diluted blood-samples examined, but unfortunately we have no quantitative observations on ox-blood.

II. EXPERIMENTS ON DOGS' BLOOD. Samples containing on an average 90 cc. of blood from healthy dogs were prevented from clotting by addition of an adequate amount of citrate of sodium in substance. Only 0.07 to 0.1 cc. of a 1 per cent watery solution of vital red was added to the sample. The colored as well as the uncolored plasma were examined spectroscopically in order to detect a possible hemolysis. The determinations were carried out about 1 to 2 hours after the blood being shed. In all regards the same method was used and the same routine was followed as in the case of ox-blood. The results are given in table 4 and figure 3.

TABLE 3

DATE	BLOOD-VOLUME	VITAL RED	HEMATOCRIT	PLASMA VOLUME		
				Calculated	Determined	Error on the determination
	cc.	*cc.*	*per cent*			*per cent*
25/9	298	0.40	29*	212	242	14.0
	450	0.60	29	329	375	14.0
3/10	320	0.45	28	230	265	15.0
25/9	300	0.40	24	228	250	10.0
3/10	355	0.50	21	280	294	5.0
13/9	200	0.30	20	160	176	10.0
17/9	196	0.30	17	167	182	9.0
3/10	300	0.45	15	255	281	10.0
1/10	326	0.49	14	280	306	9.0
17/9	206	0.30	12	181	188	4.0
13/9	290	0.45	10	261	265	1.5
12/9	190	0.30	10	171	167	−2.0
17/9	299	0.45	9	272	272	0.0

* This sample was, owing to break on the centrifuge, centrifuged only in 45 minutes; the figure 29 is, therefore, not quite reliable.

Apart from the experiment on 28/1 which for unknown reasons deviates far beyond the limits of error of the method, it is evident that also the determinations on dogs' blood involve an error going in the same direction as in the case of ox-blood and in part due to the same sources of error, but far less pronounced. It is seen also that the mutual agreement of the determinations is less than is the case in the above mentioned determinations on ox-blood in spite of the fact that we were now rather more familiar with our experimental technique. The disagreement may as mentioned above be due partly to the relative smallness of the blood samples, yet we must assume that the mutual deviations at least partly are due also to differences which must be sought in the blood itself.

The curve given in figure 3 must of course be somewhat arbitrary, never-

theless it is probable that it is in the main correct, the more so, as it has the same bend as we find in the curve figure 2, representing the determinations on diluted ox-blood. The dog-blood was certainly undiluted, but the rate of precipitation of the red cells in this blood was as a rule even greater than in the diluted ox-blood and enormously much greater than in undiluted ox-blood. The rate of precipitation of the red cells was ascertained by estimating the layer of clear plasma formed in one hour and a half (the blood-sample was standing in a usual measuring glass), and expressed in per cent of the total height of the blood-column. As indicated in table 4 we found, in the 12 blood samples examined, in 9 samples a rate of precipitation varying between 0 and 6, on an average about 3 per cent. In three samples, however, the rate was essentially increased, namely, to 38, 12.5 and 23 per cent, and these 3 samples gave errors on the plasma determination of +12, +18 and −3 per cent respectively. It is very improbable, therefore, or at least very doubtful, whether a correlation between the error on the plasma determination and the rate of precipitation of the red cells really exists.

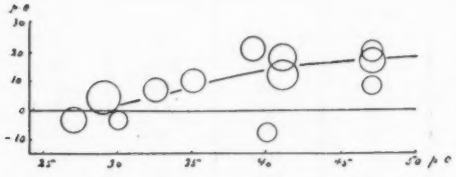


Fig. 3. Experiments on dog's blood. Abscissa: Volume of the red cells. Ordinate: Error on the plasma determination.

It may be added that also when dog-blood is examined hemolysis gives rise to considerable irregularities; these, however, do not always go in the same direction.

III. Experiments on human blood. The very considerable errors on the plasma determination found in ox-blood and in dog-blood rendered the examination of the blood of man necessary. Indeed, it proved difficult to obtain samples of human blood sufficiently large to secure a reliable determination. Moreover the samples obtained were prevented from clotting by means of sodium citrate in solution and the blood was thus—though only to a slight degree—diluted. Only 6 of the samples examined contained 100 cc. of blood or more; these are tabulated in table 5. In figure 4 the results of 18 determinations on blood from female subjects are shown graphically in the manner already described; the six above named determinations are marked on the tracing by hatching of the circular area; coincidence of two hatched fields is indicated by an entirely black area. The sample containing only 5 per cent of red cells was a filtrate from a partly coagulated blood-portion. The apparently great dispersion of the results may be explained by the increasing influence of the errors estimated on page 506 owing to the smallness of the blood-samples examined. In

fact only one determination (the one at the abscissa 30) seems to indicate a real deviation from the true value, and it must be emphasized that the results of the 6 most reliable experiments fall on the zero-line. As, however, none of the samples contained more than 36 per cent of red cells it is possible, though not probable, that an augmentation in the number of erythrocytes would involve an error on the determination going in the

TABLE 4

DATE	BLOOD-VOLUME	VITAL RED SOLUTION	HEMATO-CRIT	RATE OF PRECIPITATION	PLASMA VOLUME		
					Calculated	Determined	Error on the determination
	cc.	*cc.*	*per cent*	*per cent*	*cc.*	*cc.*	*per cent*
20/1	85	0.07	47.0	4.9	45.0	54.0	20.0
30/1	90	0.075	47.0	0	48.0	52.0	8.5
3/2	84	0.07	47.0	>0	44.5	52.0	17.0
8/1	126	0.10	41.0	38.0	74.0	83.0	12.0
16/1	82	0.07	41.0	12.5	48.0	57.0	18.0
28/1	88	0.09	40.0	4.6	53.0	49.0	−7.5
9/1	113	0.10	39.0	3.6	69.0	83.5	21.0
29/1	85	0.07	35.0	6.0	55.5	61.0	10.0
17/1	90	0.09	32.5	1.7	60.0	64.0	7.0
10/1	92	0.10	30.0	4.6	64.5	62.5	−3.0
30/1	64	0.05	29.0	3.2	45.5	47.5	4.5
16/1	68	0.06	27.0	23.0	49.5	48.0	−3.0

TABLE 5

DATE	BLOOD-VOLUME	VITAL RED SOLUTION	HEMATOCRIT	PLASMA VOLUME		
				Calculated	Determined	Error on the determination
	cc.	*cc.*	*per cent*			
24/11	239	0.30	36.0	153.0	154	0.65
	232	0.30	36.0	148.0	150	1.3
17/11	99	0.10	32.0	67.5	67	−0.75
	119	0.10	31.5	81.5	83	1.8
31/1	124	0.15	27.0	90.5	91	0.55
15/12	121	0.10	5.0	115.0	118	2.6

same direction as in the blood from cattle and dogs; but at all events, an error of this kind cannot rise to such a height as to be deleterious to the clinical appliance of the method.

The rate of precipitation of the red cells is even greater than in the case of dog's blood; but also in human blood no correlation can be detected between the error on the plasma-determination and the rate of precipitation in the single experiment. The rate of precipitation (in human blood

determined after standing in only 1 hour) in 8 of 9 samples examined ranged from 8 to 15 per cent being on an average 12.5 per cent; in one case in which the error was 0 it was only 2 per cent and in the only sample in which the error was considerable the rate of precipitation was 13.

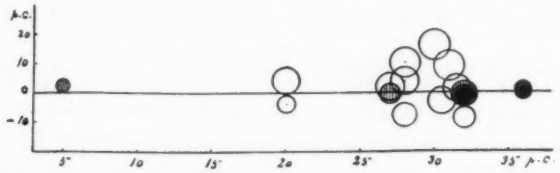


Fig. 4. Experiments on human blood. Abscissa: Volume of the red cells. Ordinate: Error on the plasma determination

Also on human blood it was tried to repeat the determination on the same blood-portion by adding a further amount of dye-solution to the colored blood. The results of 2 such experiments are given below.

TABLE 6

DATE	BLOOD-VOLUME	VITAL RED SOLUTION	HEMATOCRIT	PLASMA VOLUME		
				Calculated	Determined	Error on the determination
	cc.	*cc.*	*per cent*			
17/11 I	99	0.10	32.0	67.5	67.0	−0.75
	92	0.10 more	32.0	62.5	64.5	3.2
II	119	0.10	31.5	81.5	83.0	1.8
	100	0.10 more	31.0	69.0	67.0	−2.9

These results prove that the dye-methods may be used for repeated blood-volume determinations in man. No new sources of error are introduced, nor are the calculations more complicated because of the repeated addition of dye-substance. The necessary and sufficient condition is that we always have plasma (uncolored or weaker colored) for comparison.

SUMMARY

A report of test-experiments regarding the dye-methods for determining the blood-volume is given.

In all the experiments recorded vital red has been used. Congo red has been tried but proved to give very unreliable results because of the color being altered in character, when the dye-substance is mixed with the blood-plasma. As a rule the shade of color was altered to such a degree that the colorimetry was rendered quite illusory.

Hemolysis causes very serious errors on the colorimetric determination.

It is shown that bloods from different species behave in a quite different manner.

In undiluted ox-blood we find a very great systematic error, strictly proportional to the volume of the red cells. When the blood is diluted the error is diminished beyond the proportion which might be explained by the simultaneous decrease in the relative volume of the red cells, being zero, when the latter is reduced to about 10 per cent. The curve connecting the hematocrit-reading with the error on the plasma-determination is not as in the case of undiluted blood a straight line. It is shown that dye-substances are adsorbed to the red corpuscles.

In undiluted dogs' blood we find an error on the plasma determination likewise increasing, though not proportionally, with increasing volume of the red cells. When the error in per cent is plotted as ordinate against the hematocrit-reading as abscissa we get a curve almost identical in shape with that obtained from the experiments on diluted ox-blood, but the point of intersection with the abscissa is displaced considerably to the right, the error being zero, when the hematocrit-reading is about 30 per cent.

In samples of human blood from female subjects, slightly diluted by adding citrate of sodium in solution, no systematic error could be detected; yet it must be borne in mind that none of the blood-samples contained more than 36 per cent of the red cells.

It is shown that dye-solution may be added repeatedly to the same sample of blood without any inconvenience; when only a sample of blood is drawn for control before each injection of dye-solution the experiment as well as the calculation is carried out in the usual manner.

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XVI. THE EFFECT OF ADRENALIN UPON THE CORONARY CIRCULATION

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If adrenalin has an effect on the coronary vasculature we must assume from our present knowledge as to the point of action of this drug that these vessels are innervated by sympathetic vasomotor nerves. Both the innervation of the coronary vessels and the effect of adrenalin upon them is still imperfectly understood. Many investigators cling to the older idea that those organs, so called essential to the vital activity of the animal, the heart, brain and lungs, are innervated either by vaso-dilator nerve fibers so that adrenalin produces vaso-dilatation only, or that they possess no vasomotor nerve fibers at all. The vasomotor phenomena observed upon stimulation of the vagus and accelerator nerve trunks have been variable and inconstant due to the changes in the cardiac activity produced.

As late as 1906 Bayliss (1) maintained that there was no evidence of vasomotor fibers in the vagus or in the accelerator nerves to the heart. Panum (2), Porter (4), Porter and Bayer (5), Maass (6), Morawitz and Zahn (7) and Wiggers (8) believed they were able to demonstrate that the vagus nerves contain vaso-constrictor fibers to the coronary arteries, while Martin (3), Meyer (9) and Drury and Smith (34) noted only dilatation of the coronary vessels upon stimulation of the vagus nerves.

The results upon sympathetic nerve stimulation are as variable and inconclusive as are those obtained upon vagus nerve stimulation. Martin (3) found no vasomotor effect upon stimulation of the accelerator nerves. Maass (6) and Brodie and Cullis (11) conclude that the coronary vessels are supplied by both vasoconstrictor and vaso-dilator fibers from the sympathetic system whereas Dogiel and Archangelsky (10) noted only vaso-constriction and Morawitz and Zahn (7) only vaso-dilatation upon stimulation either of these nerves or of any of their component parts.

The published observations on the effect of adrenalin upon the coronary vessels fall into three distinct groups according to the methods of investigation used, i.e., *1*, those in which the drug was injected intravenously into the animal; *2*, those in which the excised coronary vessels were bathed in some medium to which the adrenalin was added; and, *3*, those in which the excised heart was perfused with some isotonic solution to which the drug was added.

The increased coronary flow noted by Bond (12), Morawitz and Zahn (13), (14) and Meyer (15) after the intravenous injection of adrenalin is the result of two conditions, first the increased aortic pressure and a consequent passive dilatation of the coronary arteries, and second, by a true coronary vaso-dilatation independent of the increased systemic blood pressure.

Langendorff (16), Eppinger and Hess (17), DeBonis and Susanna (18), Pal (19), Janeway and Park (20), Cow (21), Park (22) and Campbell (23) all agree that adrenalin produces relaxation of the excised coronary ring of the ox when added to the solution in which the ring is suspended. Campbell (23) and Barbour (24) noted similar changes in the excised coronary vessels of the sheep and calf respectively. Using rings made from the human coronary artery Barbour (24) observed only contraction in response to the extract.

The results obtained upon the injection of adrenalin in perfused excised hearts are complicated by a number of factors; those of mechanical origin, increased heart rate and force, and those of chemical origin, increased metabolites.

Schafer (26) maintained in 1899 and again in 1904 that adrenalin tends temporarily to increase the flow of blood in the coronary vessels, owing to the increase in rate and force of the heart beat. A few years later Elliott (27) noted increased rate of coronary flow independently of muscular metabolites. A similar vaso-dilatation of the coronaries after the addition of adrenalin to the perfusing fluid was reported by Evans and Starling (29), Markwalder and Starling (30) and Krawkow (31). Wiggers (8), Drury and Smith (34) and Drury and Sumbal (35) report only vaso-constrictor responses of the coronaries to adrenalin. Brodie and Cullis, Rabe (28) and later Krawkow (32) noted both vaso-dilatation and vaso-constriction of the perfused coronary arteries upon the addition of adrenalin to the perfusion fluid. Some of Krawkow's observations (32) were made upon perfused human hearts. Barbour and Prince (33) using commercial adrenalin chloride always noted vaso-dilatation of the coronaries in rabbits and vaso-constriction in monkeys when the substance was added to the perfusion fluid. These observations supported Barbour's (25) previous findings on the vessels of sheep, pig and human, and they therefore concluded: "There exists, we believe, between certain primates on the one hand and certain of the lower mammalia on the other, a previously unsuspected difference in innervation," etc.

This investigation was undertaken to correlate if possible these contradictory facts reported by various investigators and make, if possible, a final conclusion as to the sympathetic vasomotor innervation of the heart.

METHOD. The experimental animals used were cats, rabbits and rats.

The hearts were excised either under ether anesthesia or cerebral concussion and exsanguination. A variety of perfusion fluids were used in these experiments, such as Locke's solutions with pH 6.2, 7, 7.4, 7.6 and 8.2, to which usually autogenous defibrinated blood 2 per cent was added; Locke's solution pH 7.6, to which hirudinized blood was added to 2 per cent; saline pH 7.6 to which calcium chloride of sufficient quantity was added to make the same concentration as that found in Ringer's solution; saline pH 7.6 to which potassium chloride was added making the same concentration of potassium as found in Ringer's solution and finally Ringer's solution alone of pH 7.6.

The method of perfusion was essentially the same as that used by Sherrington and Sowton (36) in perfusing the heart, with such minor changes as previously reported by one of us (37). The free end of the arterial cannula was connected to a short rubber tube leading from a reservoir containing the modified Locke's solution. The perfusion pressure in this series of experiments varied from 45 to 90 mm. of mercury but was uniform throughout each experiment.

The heart was perfused by inserting a cannula in the aorta near the organ. Although the accuracy of this method has been criticised by Wiggers (8) and Schafer (26) we believe, as Dale (38) found, that with small hearts the defects mentioned do not seriously disturb the average rate of outflow. In a few instances the heart was immersed in the perfusion fluid in a glass cylinder. The excess fluid, as it escaped from the heart, drained off and dropped on a receiving tambour connected by rubber tubing to another tambour which recorded the rate of perfusion in drops. The heart was held with its apex upward, the apex being connected by a ligature to a myograph, thus recording the changes in cardiac activity on the kymograph surface. For all the remaining experiments a 100 cc. pyrex beaker was inverted over a funnel through which the perfusion fluid from the heart dropped on the lever of the receiving tambour. The perfusion fluid to the heart ran through a cannula fused in the side of the inverted beaker. In the top of the beaker a small opening was made to permit free passage of the ligature from the apex of the heart to the myograph. Free drainage of the organ was obtained at all times by having its apex upward. The perfusion rate was recorded as previously stated.

The preparations of adrenalin employed were adrenalin (alkaloid) and adrenalin chloride (Parke, Davis & Co.), suprarenalin (Armour's) and synthetic "suprarenium" (Meister, Lucius & Brüning). The solid preparations dissolved in the perfusion mixture and the liquid preparations usually undiluted and always at the same temperature as the perfusion fluid were injected into the perfusate near the arterial cannula against the perfusion fluid flow. Such injections had no lasting effect upon the perfusion pres-

sure as this was quickly equalized by the escape of oxygen through the mercury valve.

The adrenalin solutions without corrections as measured, would range in dilution from 1:1000 to 1:10,000,000. As will be shown later because of the chloretone in adrenalin chloride and suprarenalin, adrenalin (alkaloid) and the synthetic adrenalin were exclusively used in this work. The powder was carefully weighed and added to the necessary amount of the perfusion fluid at 37.5°C. to make about a 1:1000 dilution. One cubic centimeter of this mixture while being agitated was then added to 9 cc. of the perfusion fluid, etc., in making the dilutions. Inasmuch as the alkaloid and the synthetic preparations are comparatively insoluble, and in the higher concentrations leave some undissolved material these solutions were filtered before they were injected. Colorimetric estimations (39) were also made as to the concentration of dissolved adrenalin of some of the dilutions. It was found that the 1:1000 dilution varied from 1:1300 to 1:2900; the 1:10,000 from 1:7,500 to 1:8,700; and the 1:100,000 from 1:50,000 to 1:66,600. These corrections for the dilution due to the solubility of the adrenalin are taken into consideration throughout.

The effects of chloretone of the same concentration as that found in adrenalin chloride dissolved in normal saline and the fluids of low and high pH were also tested.

A chronograph marking fifteen second intervals was placed at the zero pressure level of the perfusion fluid. Unless otherwise stated all writing points were placed one above the other.

Results. Though our results upon the injection of adrenalin (alkaloid) or of synthetic adrenalin into the perfusate of excised hearts of rabbits, cats and rats are variable there appears to be a definite relationship between concentration of the drug and effect produced. In table 1, it can be seen that in hearts perfused with blood Locke's solution, adrenalin of high dilution has a much greater tendency to produce coronary vaso-dilatation than vaso-constriction, and that in low dilution adrenalin almost always produces vaso-constrictor effects. These results were obtained in both active and inactive hearts in all species of animals so tested.

If, however, these organs are perfused with Ringer's solution containing no blood the vaso-constrictor effect predominates. Similar observations are recorded when a perfusion fluid of Ringer's solution from which either potassium or calcium is omitted is used and adrenalin injected. Adrenalin chloride, suprarenalin and chloretone produce vaso-dilator effects almost always even in concentrated solutions.

Active heart. *The effect of adrenalin (alkaloid and synthetic) on coronary circulation in the active heart.* Inasmuch as increased coronary flow

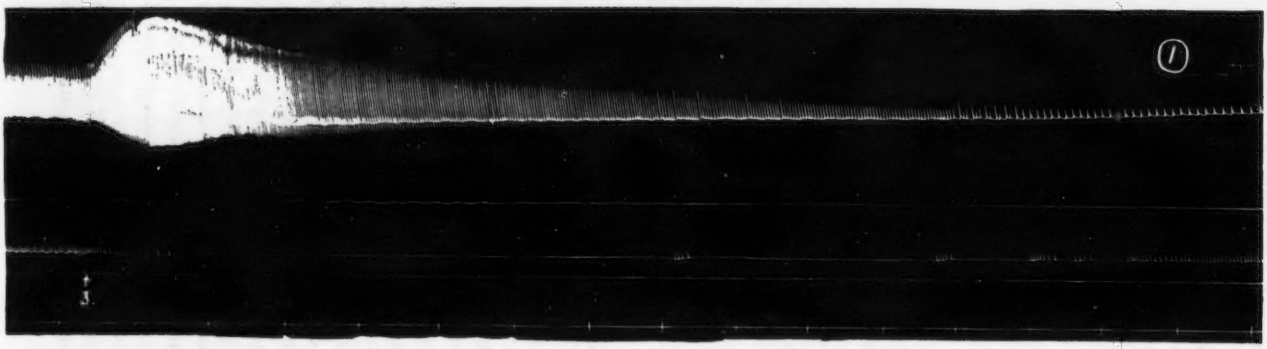

Fig. 1. Excised rabbit heart perfused with Locke's solution pH 7.4 modified by the addition of defibrinated autogenous blood to 2 per cent. Top record is of cardiac activity, immediately below it the perfusion pressure in millimeters of mercury. Bottom record is the time marked in 15-second intervals and zero perfusion pressure and above it the point of injection. Middle record is that of the rate of flow of perfusion fluid in drops from the heart.

3. One cubic centimeter adrenalin (alkaloid) 1:1,300 to 1:2,900 solution (Blood-Locke's pH 7.4 filtered). See text.

TABLE 1

TABLE 1

A table showing the effect of different concentrations of adrenalin (alkaloid and synthetic), adrenalin chloride and chloretone upon the coronary flow in excised perfused hearts, of cats, rabbits and rats. The perfusion pressure varied from 45 to 90 mm. mercury pressure but was constant for each experiment. Both the perfusion flow and the drug to be injected were kept at 37.5°C. The hearts of 33 cats, 35 rabbits and 4 rats were employed. The results of both active and inactive hearts are tabulated. One cubic centimeter of the solution was injected in each case.

PERFUSION FLUID	ADRENALIN 1:5,000,000–1:6,660,000				ADRENALIN 1:500,000–1:666,000							ADRENALIN 1:50,000–1:66,600								ADRENALIN 1:7,700–1:8,700								ADRENALIN 1:1300–1:2900							ADRENALIN CHLORIDE 1/1000–1 cc.							CHLORETONE 2½ GRAINS PER FLUID OUNCE			
	Active heart	Inactive heart			Active heart			Inactive heart				Active heart			Inactive heart				Active heart			Inactive heart					Active heart		Inactive heart					Active heart		Inactive heart					Active heart	Inactive heart		
	+−	+	+−	−	+	+−	−	+	+−	−	0	+	−+	+−	−	+	+−	−	0	+	+−	−	+	−+	+−	−	0	+	−+	+	−+	+−	−	0	+	−+	+	−+	+−	−	0	+	+	−	0
Blood-Locke's solution,																																												
pH 6.2		2	1		2	4		3		3	1	1	1	1		2	2	3	3	2	4	2	3		1		1	2		1	1	1	7	1		4	1	3			5		
pH 7.0								1		2						2		1								1										4							
pH 7.4												3		1	1	1		2	3	4		4	1			3	1	1	8				9	1	4		7					8		
pH 7.6		1				4	4	3		3	1	4		4		1	1	6	6	2	2		8	1	2	6	2	2	5	5			15		3	2	20			1		2	4		
pH 8.2		1			4			1	1		1	3		2		1				2	1		2		1	1	1	2	2			3	3	1	2		2					2	3		
Total number of observations		4	1	0	6	8	4	6	1	8	3	11	1	8	1	7	3	12	12	10	7	6	14	1	4	10	6	7	15	6	1	4	35	3	9	2	37	1	3	1	0	4	20	0	0
Ringer's solution,																																												
pH 7.6	4			2				8	5	11	1					14	4	10	3				2			4	3			5		2	28	2			22			1					
pH 7.6—K																6		14	6				3			9	5			2		11	9	2			17		1	2				2	2
pH 7.6—Ca																2		11	14				2			14	4					1	21	1			8		1	3	6		7		

+ = increased coronary flow.
+− = increased coronary flow followed by decrease.
−+ = decreased coronary flow followed by increase.
− = decreased coronary flow.
0 = no effect.

is most common in active hearts, and only a limited number of records can be presented, these shall be omitted in the presentation. However, marked coronary vaso-constriction in an active heart is sometimes obtained and figure 1 is presented as an example. In this figure a rabbit heart was perfused with warm oxygenated blood Locke's solution pH 7.4 at a pressure of 82 mm. of mercury. Previous injections of adrenalin theoretically 1:100,000 and 1:10,000 doubled the force of contraction, increased the rate of contraction and coronary flow. At *3* in the curve presented 1 cc. of a 1:1000 adrenalin in blood Locke's solution, at the same temperature as the perfusate, was injected into the perfusion fluid. It should be recalled that the concentration of adrenalin here was not 1:1000, but probably somewhere between 1:1300 and 1:2900. There resulted as in the two previous injections marked increase in rate and force of contraction but

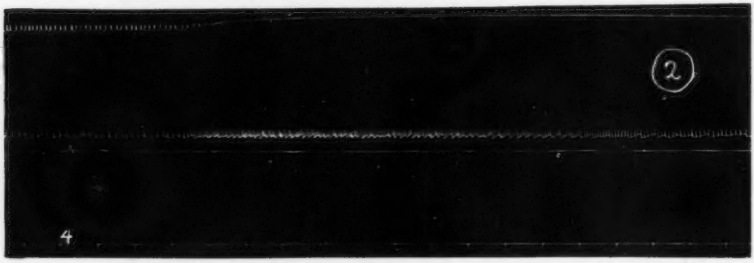

Fig. 2. Excised rabbit heart perfused with Locke's solution pH 7.6 modified by the addition of defibrinated autogenous blood to 2 per cent. Top record cardiac activity and below it rate of perfusion in drops leaving the heart. Bottom record the time in 15 seconds, point of injection and zero perfusion pressure and above it the perfusion pressure in millimeters of mercury.

4. One cubic centimeter adrenalin chloride 1:1,000 injected.

instead of increased coronary flow an almost complete stoppage resulted. This decreased coronary flow or decreased nutrition to the heart, gradually brought about decreased rate and force of contraction; however, after the effect of the injection had worn off the rate of coronary flow became as rapid as it previously had been. A large number of such records were obtained in different hearts. See table 1.

The effect of adrenalin chloride, suprarenalin, chloretone and acid upon coronary circulation in the active heart. Figure 2 shows quite the opposite results upon injecting adrenalin chloride 1 cc. of a 1:1000 solution. In this instance another rabbit heart was perfused with warm oxygenated Locke's solution pH 7.6 to which was added autogenous defibrinated blood to 2 per cent. The perfusion pressure was 64 mm. mercury. Previous injections of adrenalin (alkaloid) had produced vaso-dilatation in dilute

solutions and vaso-constriction with concentrated solutions. In this instance complete stoppage of the heart beat resulted. The rate of perfusion flow increased from 6 to 26 drops per fifteen seconds. A dilatation persisted for about three and a half minutes. In some instances increased

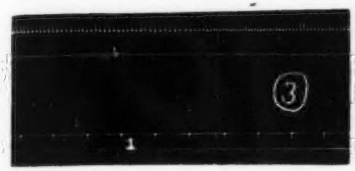

Fig. 3

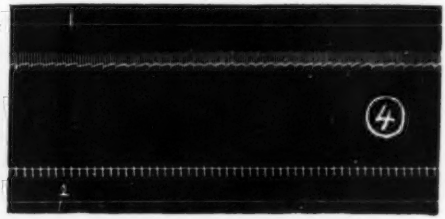

Fig. 4

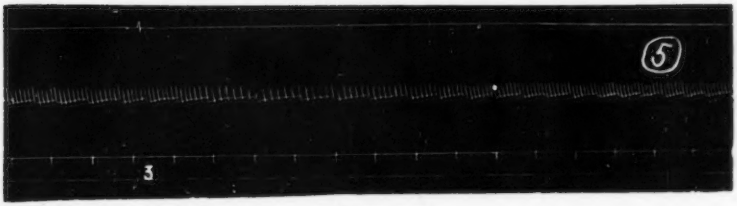

Fig. 5

Fig. 3. Inactive excised cat's heart perfused with Ringer's solution pH 7.6 to which autogenous defibrinated blood was added. Top record, perfusion rate in drops; next below, the perfusion pressure in millimeters of mercury; bottom record the point of injection and above it the time interval in 15 seconds and zero perfusion pressure.

1. One cubic centimeter adrenalin (1:50,000–1:66,600).

Fig. 4. Unless otherwise stated in this and all of the following records the inactive excised perfused rabbit's heart was used. The top record, the perfusion pressure in millimeters of mercury; below it the rate of flow of perfusion fluid in drops leaving the heart; the bottom record the point of injection of the drug and above it the time interval in 15 seconds and zero perfusion pressure.

Locke's solution pH 7.6 modified by the addition of autogenous defibrinated blood 2 per cent.

1. One cubic centimeter adrenalin (1:50,000–1:66,600) solution See text.

Fig. 5. See figure 4. Ringer's solution pH 7.6.

3. One cubic centimeter adrenalin (synthetic) (1:1,300–1:2,900).

activity of the heart resulted but in the majority it was decreased. In many instances the same quantity of chloretone as that found in the adrenalin chloride solution (2¼ grains to the ounce of normal saline) had a similar action upon coronary flow and activity of the heart. See table 1. This confirms Wiggers' (8) findings on the effect of chloretone upon the

coronary circulation. In addition acid solutions with a pH similar to that found in adrenalin chloride consistently brought about vaso-dilator changes. These latter results confirm Markwalder and Starling's (30) observations on the effects of acid and alkaline perfusates upon these same organs and Gaskell's (40) observations upon the effect of alkali (NaOH) and lactic acid upon the blood vessels of the frog.

INACTIVE HEART. *The effect of adrenalin (alkaloid and synthetic) on the coronary circulation in the inactive heart.* With the use of an inactive heart the vasomotor results were more clear cut. Either vaso-dilatation or vaso-constriction could be obtained by the choice of the dose injected. Figures 3, 4 and 5 demonstrate clearly the effect. In figure 3 an excised cat's heart was perfused with Locke's solution, pH 7.6, to which had been added autogenous defibrinated blood 2 per cent. The perfusion pressure was 54 mm. of mercury. At *1*, 1 cc. of about 1:50,000 solution adrenalin (alkaloid) dissolved in blood Locke's solution was injected into the perfusate. There resulted an increased rate of perfusion flow from 8 to 12 drops per fifteen seconds. In figure 4 a rabbit heart was similarily perfused. The perfusion pressure was in this instance 78 mm. of mercury. At *1*, 1 cc. of the same strength solution of adrenalin (alkaloid) dissolved in blood Locke's solution was injected. The perfusion flow leaving the heart increased from 11 to 14 drops per minute.

In figure 5, a rabbit's heart was perfused with warm oxygenated Ringer's solution, pH 7.6 at a pressure 70 mm. of mercury. At *3*, 1 cc. of a 1:1,300–1:2,900 synthetic adrenalin, in Ringer's solution, was injected into the perfusate. There resulted very shortly a decrease in the rate of the perfusion flow from 9 to 5 drops per fifteen seconds, which was later followed by an increase to 11 drops for the same interval of time. Similar results are given in table 1. These results were obtained in hearts perfused with Locke's solution to which defibrinated blood was added as well as in hearts which were perfused with Ringer's solution. When suprarenalin was injected into the perfusate, Rabe (28), as said before, found in the same heart vaso-constriction with Ringer's solution as the perfusate but vaso-dilatation when Ringer's solution to which 5 to 10 per cent blood had been added was used instead of the whole Ringer's solution. In our experiments in which Ringer's solution alone was used only vaso-constriction was obtained even with dilutions as high as 1:6,660,000.

Adrenalin chloride and suprarenalin. These drugs had the same effect on inactive as on active hearts. In almost all cases vaso-dilatation occurred.

ADRENALIN ACTION UPON THE CORONARY CIRCULATION IN HEARTS PERFUSED WITH RINGER'S SOLUTION OMITTING EITHER CALCIUM OR POTASSIUM. *The effect of calcium and potassium upon coronary circulation.* In 1911 Hooker (41) found the vascular tone of perfused blood vessels of the

frog to be increased by the addition of calcium chloride to normal saline and to be decreased by the addition of potassium chloride. It was pointed out by Pearce (42) and by Bergengren (43) that calcium plays an important rôle in the perfusion fluid, that of retaining the sympathetic irritability to adrenalin.

We found upon perfusing cat and rabbit hearts with a solution containing the same elements as found in Ringer's solution, but the potassium

TABLE 2

A table showing the effect of the chemical elements calcium and potassium in the perfusion fluid which otherwise would have been Ringer's solution upon the coronary flow. pH of fluid 7.6 in all instances with the pressure and temperature the same. Temperature 38.5°C.

DATE, 1925	EXCISED HEART PERFUSED	RATE OF CORONARY FLOW IN DROPS PER 15 SECONDS	
		Ringer's solution, omit potassium pH 7.6 ($NaCl + CaCl_2 + NaHCO_3 + H_2O$)	Ringer's solution, omit calcium pH 7.6 ($NaCl + KCl + NaHCO_3 + H_2O$)
April 7	Rabbit	14.0	30
		1.5 ←	30
8	Rabbit 1	0.33	9
8	Rabbit 2	0.2	18
8	Rabbit 3	11.0	40
9	Rabbit	1.75	28
		6.0 ←	28
		6.0	9
10	Cat 1	4.0	35
		4.0 ←	12
10	Cat 2	0.25	32
14	Cat	2 0	16
25	Rabbit	4.0	14
27	Rabbit	3.0	9
28	Rabbit	3.0	14
29	Rabbit	4 0	24
30	Rabbit	11.0	16
May 2	Rabbit	4.0	16
5	Cat	4.0	12
Average		4.42	20.6

chloride omitted, that the rate of flow of the perfusion fluid was always slow and the contractions of the heart quickly ceased. If, however, this solution was replaced by one containing potassium chloride instead of the calcium chloride the rate of flow became very rapid. These solutions had a pH 7.6 in all instances. The results are tabulated in table 2. It will be seen that the average rate of flow with Ringer's solution in which potassium is omitted ($NaCl + CaCl_2 + NaHCO_3 + H_2O$) was 4.42 drops per fifteen seconds, with the potassium present and the calcium omitted

($NaCl + KCl + NaHCO_3 + H_2O$) the rate of flow in these same hearts averaged 20.6 drops in the time. These results, were obtained when either solution was used first and then replaced by the other.

Figure 6 is an example of such an experiment. In this instance a cat heart was perfused first with Ringer's solution in which the potassium was omitted, *Ca*. This was replaced by another solution at ↑ in which the calcium was omitted, *K*. The pH was 7.6 and the temperature of each 38.5°C. As will be seen in the figure the perfusion rate rapidly increased from 2 to 16 drops per fifteen seconds.

The action of adrenalin. Table 1 shows that adrenalin (alkaloid or synthetic) in even moderately dilute solutions, produces vaso-constriction in the majority of hearts tested. Figures 7, 8 and 9 show the usual effect in which both moderate and strong solutions were used in inactive hearts. In figure 7, a rabbit heart was perfused at 58 mm. mercury pressure with Ringer's solution pH 7.6, from which potassium was omitted. At *1*, 1 cc.

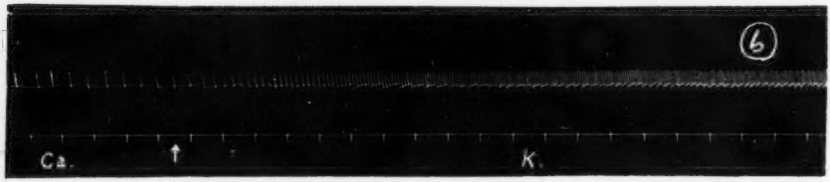

Fig. 6 Inactive excised cat's heart, otherwise see figure 4. *Ca*, Ringer's solution pH 7.6 in which potassium chloride was omitted used as the perfusion fluid. This was gradually replaced by a Ringer's solution pH 7.6 in which the calcium chloride was omitted, *K*. Temperature of each, 38.5°C.

of a 1:1,300–1:2,900 adrenalin was injected into the perfusate. The rate of perfusion fluid decreased from 4 to 2.5 drops per fifteen seconds which later increased to 5. In figures 8 and 9 the perfusion pressure was 60 mm. of mercury and the temperature 37.5°C. In figure 8, 1 cc. of a 1:1,300–1:2,900 adrenalin alkaloid and in figure 9, 1 cc. of a 1:1,300–1:2,900 synthetic adrenalin was injected. The perfusion fluid was the same in both, Ringer's solution omitting potassium. In figure 8 the rate of perfusion fluid after the injection changed from 6 to 5 to 12 drops per minute. In figure 9 the rate changed from 16 to 8 to 18 drops per minute.

The reactions of the coronary vessels to adrenalin in the absence of calcium and in the presence of potassium are markedly diminished. Either no effect or vaso-constriction only were observed after such injections. See table 1.

Adrenalin chloride and suprarenalin. When Ringer's solution in which either calcium or potassium was omitted is used as the perfusate adrenalin chloride or suprarenalin causes in most cases vaso-dilatation, these results

differing from those obtained when either normal Ringer's solution or modified Locke's solution was employed as the perfusate. See table 1.

VAGUS AND SYMPATHETIC STIMULATION UPON CORONARY FLOW. Sher-

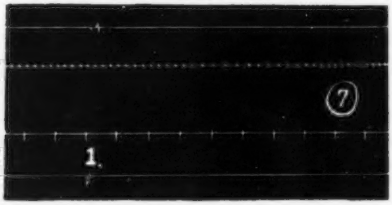

Fig. 7. See figure 4. Perfusion fluid Ringer's solution pH 7.6 modified by the omission of potassium chloride.

1. Adrenalin (alkaloid) 1 cc. (1:1,300–1:2,900) solution.

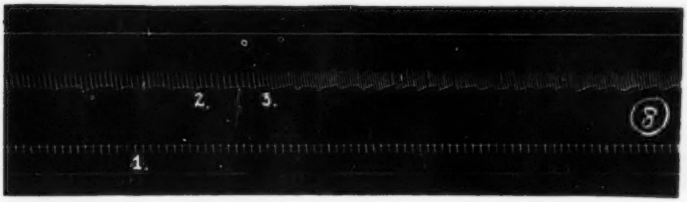

Fig. 8. Perfusion fluid and injection same as in figure 7. See also figure 4.

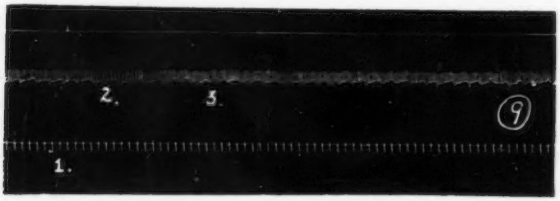

Fig. 9. Excised heart of the same animal and perfusion fluid the same as that used in figure 8. See also figure 4.

1. Adrenalin (synthetic) 1 cc. (1:1,300–1:2,900) solution.

rington (44) shielded electrodes were applied to the left pair of nerves, and varying strengths of stimuli at rates of 9 and 55 interruptions per second applied. Like Porter (5) we found no difference in the effect with differ-

ent rates of stimulation. We obtained upon vagus nerve stimulation increased coronary flow 3, decreased flow 13, decrease followed by an increase 2 and no change 13 times. Upon sympathetic stimulation there was observed an increase in coronary flow 2, decrease 7 and no change 7 times. In some instances when either nerve was stimulated we obtained increased coronary flow in which there was no visible trace of change in cardiac activity. In other instances in which the nerves were similarly stimulated only decreased coronary flow was observed although no signs of change in cardiac activity were noted. Although we obtained both increased and decreased coronary flow in one heart in which there was no activity upon vagus and sympathetic nerve stimulation we do not feel that these results justify conclusions being drawn as to vasomotor fibres in these nerves.

Discussion. We found, as did Brodie and Cullis (11), that adrenalin is capable of producing both vaso-dilator and vaso-constrictor changes in the coronary vessels of excised perfused hearts of rabbits, cats and rats. Although, in our experiments vaso-dilatation was found with all doses this change results primarily from the more dilute solutions. This has been found to be true for the vessels in skeletal muscle (45), intestine (46) and even the blood vessels of a perfused frog (43). As in other organs (43), (45), (46), (47) adrenalin in concentrated doses causes marked vaso-constriction in blood vessels.

The slowing of coronary flow as seen in figure 1 and in the results shown in table 1, cannot be due to mechanical interference as a result of the increased action of the heart. More dilute solutions in this and other hearts showed an equal increase in cardiac activity with increased coronary flow. Inasmuch as the solutions injected were filtered and the solution was taken up in the syringe from the top of the cylinder, we believe these results can hardly be attributed to undissolved adrenalin particles acting as emboli. Temperature changes have been ruled out as the source of these vasomotor reactions. This seems, therefore, to be a true vaso-constrictor phenomenon of the drug acting upon sympathetic vasomotor nerves in the heart.

Barbour and Prince's (33) inability to obtain vaso-constriction of the coronary vessels in excised perfused hearts of rabbits with adrenalin we believe to be due to the fact that they employed commercial adrenalin chloride. In our experiments with this solution as with the chloretone free preparation of liquid suprarenalin, we obtained almost exclusively vaso-dilator changes. In those hearts which were perfused with blood-Locke's solution and in which adrenalin chloride was injected three factors exclusive of the adrenalin content are involved in the reaction. In those in which suprarenalin was injected two factors are concerned in the dilatation produced. The first factor with either solution is that of viscosity.

The second factor is the acidity of the solution injected. Gaskell (40) in 1880, using the systemic vessels of the frog, and later Markwalder and Starling (30) using the coronary vessels of the rabbit showed conclusively that acids cause vaso-dilatation.

Samples of adrenalin chloride and suprarenalin were tested as to their acidity. This was done by titrating against N/10 NaOH using phenophthalein as the indicator. In these it was observed that it required an average of 0.15 cc. N/10 NaOH for each cubic centimeter adrenalin chloride and 0.19 cc. N/10 NaOH for each cubic centimeter suprarenalin to neutralize them. The acidity of the perfusion fluid is in itself sufficient to produce a marked effect on the coronary flow and is a factor to be considered in reviewing the results of all perfusion experiments reported in the literature in which commercial liquid adrenalin solutions were used. In most reports there has been no consideration taken of the vasomotor action of the preservative and this is the third factor when adrenalin chloride is used. The preservative, chloretone, produces marked dilatation of the coronaries. Wiggers (8) in 1909, in criticising Langendorff's (16) results, showed that the chloretone content of the adrenalin solution caused the relaxation of the vessel wall. Our results confirm his.

From these observations it is possible that the many conflicting opinions held concerning the effect of adrenalin solutions, not only upon the heart but in other organs also may be due in part if not wholly to the different preparations of adrenalin with their varying degrees of acidity and their preservatives. Had the pure amorphous adrenalin been used by all investigators more uniform results would have been obtained.

Our results with the use of perfusion fluids with varying pH show no marked change in the reaction of the coronary vessels to adrenalin. This may be due to the fact that we added defibrinated blood to all of these solutions. As has been shown by a large number of investigators (48) blood serum produces marked vaso-constriction which may have been instrumental in preventing the effect of changes in vascular reaction to adrenalin in the different pHs as found by a number of investigators (49). There was, however, a noticeable difference between the results obtained when the heart was perfused with Ringer's solution pH 7.6 and Locke's solution containing defibrinated blood. This difference we believe to be due to the difference in the tonicity of the vessels. In those cases in which Ringer's solution alone was used the tone was low and a greater tendency toward vaso-constriction was observed. Rabe (28) noted a similar response. The effect of tonus on the vascular response to adrenalin was also observed with other solutions and especially with Ringer's solution in which the calcium was omitted. When the heart was being perfused with Locke's solution modified by the addition of defibrinated blood, and the perfusion rate was rapid, vaso-constriction was the

usual result but if the rate of perfusion was slower dilatation could be obtained with higher concentrations. This same change was noted in the coronary response to adrenalin chloride when the heart was perfused with a Ringer's solution in which the potassium salt was omitted, and in some instances with Ringer's solution pH 7.4 in which the adrenalin chloride solution was very dilute. We believe these results confirm those of Brodie and Cullis (11) in which adrenalin chloride was used, and normal saline, and saline plus potassium were used as the perfusates. The dilatations observed with the more concentrated solutions of adrenalin chloride are probably due to the increased acidity of the solution injected and not to the increased adrenalin content. Our results also support the findings of Cannon and Lyman (50) on the dependence on vascular tone of the blood pressure response to adrenalin, and those of Gruber (45) and Dale and Richards (45) in which it was shown that the vaso-motor responses to adrenalin in the vessels of skeletal muscle were dependent upon the tonicity of the vessel wall. They are, however, at variance with the findings of some other investigators (51) on this same question, whose work is now being repeated in this laboratory with especial attention to the factors just discussed.

That calcium either increases or preserves the irritability of the sympathetic system was noted by Pearce (42) and Bergengren (43) upon systemic vessels of the frog and our results upon the coronary circulation support their findings. In most instances the calcium present appears either to heighten the irritability of the vaso-constrictor fibres or to lower the irritability of the vaso-dilator fibres of the sympathetic system to adrenalin. This was demonstrated with such active dilator substances as suprarenalin and adrenalin chloride with their decreased pH which when injected into the perfusate, Ringer's solution pH 7.6, had predominant vasodilator action. On the other hand with Ringer's solution in which potassium was omitted the relative number of vaso-constrictor responses was increased.

We believe the increased number of constrictor responses observed after the injection of adrenalin and adrenalin chloride when the heart was perfused with Ringer's in which the calcium was omitted to be due to the decreased tonus of the coronary vasculature.

SUMMARY

1. Adrenalin (alkaloid and synthetic) in dilute solutions causes vasodilatation of the coronary vessels in both active and inactive hearts of rabbits, cats and rats.

2. Adrenalin (alkaloid and synthetic) in concentrated solutions causes vaso-constriction of the coronary vessels in the hearts of these same animals.

3. Adrenalin chloride and suprarenalin produce dilatation of the coronary vessels in all dilutions and in nearly all hearts so tested.

4. The vaso-dilatation noted after the injections of adrenalin chloride and suprarenalin is not due to the adrenalin content but due to the decreased viscosity (in some cases), preservative chloretone in adrenalin chloride, and principally if not wholly to the acidity of the solution.

5. An average of 0.15 cc. N/10 NaOH was required to neutralize 1 cc. adrenalin chloride (Parke, Davis & Co.) and 0.19 cc. N/10 NaOH was required to neutralize 1 cc. suprarenalin (Armour).

6. Ringer's solutions with decreased pH made so by the addition of either hydrochloric acid or sodium acid phosphate when injected into the perfusate of perfused hearts, cause increased coronary flow. Ringer's solution with increased pH made so by the addition of sodium hydroxid or sodium bicarbonate when injected into the perfusate decrease the coronary flow. (Confirmation of Markwalder and Starling.)

7. Chloretone, of the same concentration as that found in adrenalin chloride ($2\frac{1}{4}$ grains dissolved in 1 ounce normal saline) produces marked coronary vaso-dilatation.

8. In all perfusion experiments in which the true action of adrenalin is to be determined adrenalin alkaloid should be used without the addition of acid or preservative to the solution.

9. Calcium increases the tonus of the coronary vessels whereas potassium decreases it.

10. Calcium chloride added to normal saline with a given pH increases the responsiveness of the vaso-constrictor fibres to adrenalin. The addition of potassium chloride to normal saline with a given pH decreases the response of the coronary vessels to adrenalin.

11. Decreased tonus of the coronary vessels leads to greater vaso-constrictor responses from adrenalin and vice versa.

12. Stimulation of the peripheral end of either the left vagus or accelerator nerve at times produced coronary vaso-constriction and at other times vaso-dilatation. The number of experiments are insufficient and too variable to warrant positive conclusions.

13. From our experiments with adrenalin upon the coronary circulation we can conclude that the sympathetic nervous system supplies both vaso-dilator and vaso-constrictor fibres to the coronary circulation of the heart.

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THE RELATIONSHIP OF PHOSPHATE AND CARBOHYDRATE METABOLISM

III. The Effect of Glucose on the Excretion of Phosphate in Depancreatised Dogs

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In a previous communication from this laboratory (Sokhey and Allan, 1924) it was shown in confirmation of the work of others that the administration of glucose or insulin to fasting dogs caused striking changes in the output of urinary inorganic phosphorus. The administration of insulin resulted in an immediate reduction in the excretion of inorganic phosphate in the urine, this being in the six hours immediately following the injection, too small to measure. This was followed by a period of increased excretion, so that the total output for the day was usually increased from 25 to 35 per cent. The ingestion of glucose caused similar changes, with the difference that the onset was significantly slower. The changes were so striking as to merit further study, and in a subsequent publication (Allan, Dickson and Markowitz, 1924) the effects of administering epinephrin and phlorhidzin were noted. The injection of epinephrin caused marked changes in phosphate excretion, similar to those mentioned above, whereas the injection of phlorhidzin after a small decrease, caused a marked increase in the excretion of phosphate, so that frequently repeated administration of phlorhidzin caused a continuous excessive loss of phosphate from the body.

Inasmuch as the administration of glucose to normal dogs elicited changes that were similar, but of delayed onset, to those following injection of insulin, the question was raised by Professor Macleod whether the effect of glucose might be due to stimulation of the secretion of insulin. The present investigation was undertaken to test this hypothesis, and it has been found that the administration of glucose to depancreatised dogs has little or no effect on the phosphate excretion.

Methods. Fasting depancreatised dogs that were receiving no insulin were used. They were catheterized every three hours during the day, and the twelve-hour night urine was collected as a whole. Subsequent to each catheterization 100 cc. of water were given by stomach tube during the day, and 300 cc. at the beginning of the night period. Glucose was given

only after several days had elapsed since the last injection of insulin, because it is difficult to be certain when the effect of this hormone has passed off. The urine was analyzed for inorganic phosphate (Briggs, 1922), total nitrogen, and glucose (Shaffer and Hartmann, 1920).

RESULTS. Dog Pu, a small depancreatised collie weighing 8½ pounds, was last given food and insulin on the morning of July 6. On July 8 at 10:15 a.m. it was catheterized and the urine discarded. The results are

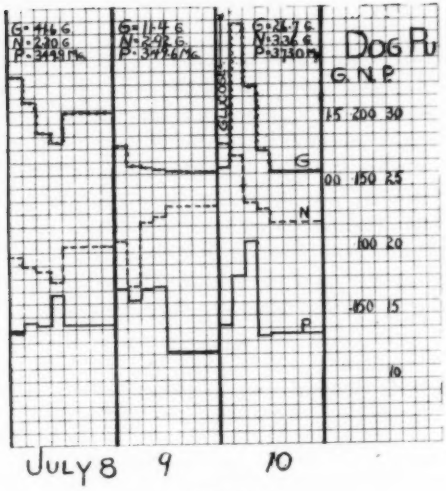
TABLE 1

Dog Pu

PERIOD	TIME	DURATION OF OBSERVATION	NITROGEN, TOTAL	NITROGEN, PER HOUR	GLUCOSE, TOTAL	GLUCOSE, PER HOUR	D/N	INORGANIC PHOSPHORUS, TOTAL	INORGANIC PHOSPHORUS, PER HOUR
		hours	*grams*	*grams*	*grams*	*grams*		*mgm.*	*mgm.*
July 8	1:15 p.m.	3.00	0.290	0.097	7.86	2.62		41.6	13.9
	4:15 p.m.	3.00	0.266	0.089	6.00	2.00		43.9	14.6
	7:30 p.m.	3.25	0.278	0.086	4.05	1.25		46.4	14.3
	10:30 p.m.	3.00	0.230	0.077	3.09	1.03		50.0	16.7
July 9	10:16 a.m.	11.76	1.239	0.105	20.06	1.75		168.0	14.3
	Total.....	24.01	2.303	0.096	41.60	1.73	18.1	349.9	14.6
	1:16 p.m.	3.00	0.326	0.109	2.94	0.980		51.4	17.1
	4:18 p.m.	3.03	0.220	0.073	1.64	0.542		49.2	16.2
	7:16 p.m.	2.97	0.369	0.123	1.42	0.478		50.7	17.1
	10:15 p.m.	2.99	0.382	0.128	1.17	0.392		51.3	17.2
July 10	10:18 a.m.	12.05	1.622	0.135	4.26	0.354		147.0	12.2
	Total.....	24.03	2.919	0.121	11.43	0.471	3.92	349.6	14.5
	1:22 p.m.*	3.07	0.563	0.183	1.50	0.489		43.8	14.3
	4:20 p.m.	2.97	0.516	0.174	11.40	3.84		53.8	18.1
	7:25 p.m.	3.08	0.425	0.138	7.24	2.35		64.3	20.8
	10:20 p.m.	2.92	0.387	0.133	2.37	0.812		39.1	13.4
July 11	10:20 a.m.	12.00	1.466	0.122	4.19	0.349		163.0	13.6
	Total.....	24.03	3.357	0.140	26.70	1.11	2.48†	373.0	15.5

* 18.4 grams glucose in 100 cc. water given by stomach tube at 1:25 p.m.
† By difference.

shown in table 1. The phosphate excretion for the 24 hours was twice as great as in normal animals, and there was no suggestion that the usual daily rhythm occurred. As the glucose excretion fell from day to day, the nitrogen excretion rose, and this is well shown by comparison of samples collected at corresponding periods on succeeding days. Thus the sample collected at 1:22 p.m. on July 10 showed a considerable increase over the corresponding sample of the day previous. The ingestion of glucose on July 10 was followed by a slight increase in phosphate excretion for the six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N=2.40 G
P=349 Mc
G=11.4 G
N=5.76 G
P=347 Mc
G=24.7 G
N=33.4 G
P=374.0 Mc
DOG PU
G. N. P.
GLUCOSE
G
N
P
15 200 30
00 150 25
100 20
150 15
10
JULY 8 9 10

Fig. 1

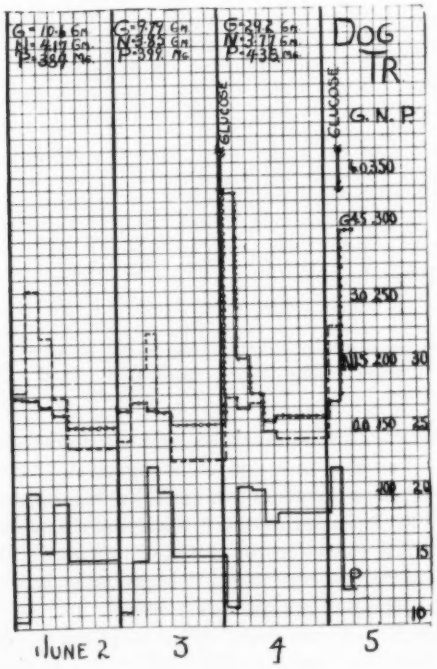
G=10.6 Gm
N=4.17 Gm
P=364 Mc
G=9.77 Gm
N=5.85 Gm
P=397 Mc
G=29.2 Gm
N=13.77 Gm
P=435 Mc
DOG TR
G. N. P.
GLUCOSE
GLUCOSE
G
N
P
0 350
35 300
30 250
15 200 30
0 150 25
20 20
15
10
JUNE 2 3 4 5

Fig. 2

hours immediately following, but a slight decrease, probably of a compensatory nature, was evident during the following six hours. The total output for the twenty-four hours was unchanged. Over 90 per cent of the ingested glucose was excreted in the urine in the first six hours. These results are also shown in figure 1 in which the vertical lines mark equal periods of three hours. The horizontal lines indicate the amounts of glucose, nitrogen and phosphorus excreted per hour. The figures give the total amount excreted in the twenty-four hours.

Dog Tr., a medium sized depancreatised fox-terrier, was last given food and insulin on the morning of May 30. On June 2, at 9:41 a.m. the animal

TABLE 2

Dog Co

PERIOD	TIME	DURATION OF OBSERVATION	NITROGEN, TOTAL	NITROGEN, PER HOUR	GLUCOSE, TOTAL	GLUCOSE, PER HOUR	D/N	INORGANIC PHOSPHORUS, TOTAL	INORGANIC PHOSPHORUS, PER HOUR
		hours	*grams*	*grams*	*grams*	*grams*		*mgm.*	*mgm*
July 22 to 23	10:30 a.m.	24.00	2.99	0.125	40.0	1.67	13.4	312.4	13.0
July 23	1:57 p.m.	3.45	0.535	0.155	4.95	1.44		65.9	19.1
	4:57 p.m.	3.00	0.474	0.158	2.88	0.096		49.0	16.3
	8:22 p.m.	3.42	0.560	0.164	3.62	1.06		50.4	14.7
	10:37 p.m.	2.25	0.443	0.197	2.78	1.23		45.2	20.8
July 24	10:45 a.m.	12.13	1.795	0.148	6.93	0.571		138.1	14.4
	Total.....	24.25	3.807	0.157	21.16	0.872	5.63	348.6	14.4
	1:33 p.m.*	2.80	0.461	0.165	1.94	0.693		58.5	20.9
	4:33 p.m.	3.00	0.750	0.250	1.82	0.607		117.1	39.1
	7:30 p.m.	2.95	0.296	0.100	9.17	3.11		52.5	17.8
	10:40 p.m.	3.17	0.116	0.0366	1.21	0.382		27.2	8.58
	Total.....	11.83	1.623	0.137	14.14	1.197		255.3	21.6

* 18.4 grams glucose given by stomach tube in 100 cc. water at 1:35 p.m. Animal weak and vomiting. Experiment terminated. Died subsequently. Pancreatectomy complete.

was catheterized, the sample discarded, and the procedures carried out as detailed above. The results are given in figure 2. The effect of giving sugar on the phosphate excretion is doubtful in this animal. Glucose was given June 4 and 5. In each case the urine in the next three hours showed the lowest phosphate excretion for the day. Although the fluctuation is within the normal diurnal variation, it is probably true that in this dog sugar caused a slight fall in phosphate excretion.

Dog Co., a small depancreatised collie weighing 9½ pounds, was last given food and insulin on the morning of July 21. In this animal the experiment had to be prematurely terminated because of the onset of profuse

vomiting. The administration of glucose resulted in a marked increase in the phosphate excretion for the next three hours. The nitrogen at first rose, and then fell to a very low level. The results are shown in table 2.

Dog N, a small depancreatised black and tan dog weighing 10 pounds, was last given food and insulin on the afternoon of August 10. On August 12, at 9:15 a.m. it was catheterized, the urine discarded, and the procedure repeated as in the above experiments. Glucose was given next day. There was no change in the excretion of phosphate. The results in this case are shown in the graphs of figure 3.

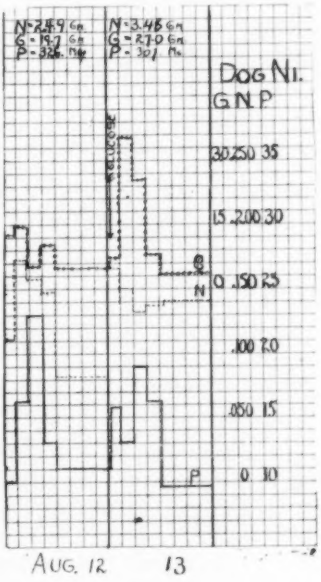

Fig. 3

Dog Lu., a small depancreatised collie weighing 11 pounds, represents a deviation from these comparatively clear-cut results. The animal was last given food and insulin on the afternoon of May 10. The administration of glucose on May 14 resulted in an immediate and marked drop in phosphate excretion for the next six hours, with no subsequent compensatory increase. As judged by the D/N ratio (obtained by difference) the animal returned all the sugar. *Post-mortem* examination demonstrated that the pancreatectomy was complete. The result is possibly due to the existence in the tissues of slight traces of previously injected insulin. The results are shown in table 3.

In five other depancreatised animals not reported in this communication because they were less completely investigated, the administration of glucose caused either no change in the phosphate excretion, or else a very slight decrease.

Discussion of results. The suggestion that the changes in urinary phosphate following administration of glucose to a normal dog are due to the ensuing secretion of insulin, is supported by the observation recorded in this paper that no significant change in the output of urinary phosphate follows the ingestion of glucose in depancreatised dogs. This conclusion is further supported by the familiar observation that the hyperglycemia which results from the administration of sugar *per os* both in man and animals,[1] is followed by hypoglycemia.

[1] Dr. Max Titso, working in this laboratory, has recently confirmed this observation in rabbits.

The explanation for the characteristic behavior of phosphate in relation to carbohydrate metabolism is apparently linked up with the problem of the fundamental action of insulin. Some attribute to insulin an amboceptor-like action in synthesizing hexosephosphoric acid. There is, however, no direct proof to support this view, and the changes in the phosphorus content of blood and urine may be due to other causes. Such an hypothesis almost necessarily presupposes that the organism cannot oxidise glucose in the absence of a hexosephosphate linkage. The results of the present investigation do not indicate that such a linkage can occur in

TABLE 3
Dog Lu

PERIOD	TIME	DURATION OF OBSERVATION	NITROGEN, TOTAL	NITROGEN, PER HOUR	GLUCOSE, TOTAL	GLUCOSE, PER HOUR	D/N	PHOSPHORUS, TOTAL	PHOSPHORUS, PER HOUR
		hours	*grams*	*grams*	*grams*	*grams*		*mgm.*	*mgm.*
May 12	1:13 p.m.	3	0.528	0.176	4.92	1.64		42.5	14.2
	4:13 p.m.	3	0.555	0.185	5.57	1.86		48.4	16.1
	7:13 p.m.	3	0.620	0.207	3.17	1.06		57.9	19.3
	10:13 p.m.	3	0.504	0.168	3.36	1.12		34.4	11.5
May 13	10:13 a.m.	12	1.285	0.107	6.00	0.50		135.7	11.3
	Total.....	24	3.49	0.141	23.02	0.96	6.60	318.9	13.3
	1:13 p.m.	3	0.557	0.189	3.36	1.12		36.2	13.1
	4:13 p.m.	3	0.478	0.159	1.86	0.62		34.8	11.6
	7:13 p.m.	3	0.343	0.114	0.83	0.28		35.0	11.7
	10:13 p.m.	3	0.349	0.116	1.20	0.40		41.2	13.7
May 14	10:13 a.m.*	12	1.112	0.092	2.90	0.24		118.1	9.8
	Total.....	24	2.84	0.118	10.15	0.423	3.58	265.3	11.0
	1:13 p.m.	3	0.348	0.116	8.16	2.72		10.6	3.5
	4:13 p.m.	3	0.516	0.172	12.00	4.00		4.69	1.6
	7:13 p.m.	3	0.382	0.127	2.80	0.93		41.0	13.7
	10:13 p.m.	3	0.391	0.130	1.41	0.47		38.3	12.7
May 15	10:13 a.m.	12	1.444	0.120	3.44	0.28		112.1	9.3
	Total....	24	3.081	0.128	27.81	1.15	3.23†	206.7	8.6
	1:13 p.m.	3	0.515	0.172	1.40	0.47		25.9	8.6
	4:13 p.m.	3	0.497	0.166	0.84	0.28		34.6	11.5
	7:13 p.m.	3	0.492	0.167	1.45	0.48		40.4	13.4
	10:13 p.m.	3	0.398	0.133	0.98	0.33		29.9	9.9
May 16	10:13 a.m.	12	1.750	0.146	0.33	0.42		123.9	10.3
	Total.....	24	3.652	0.152	9.26	0.38	2.55	254.7	10.6

* Given 18.4 grams glucose in 100 cc. water by stomach tube at 10:55 p.m.
† By difference.

the fully diabetic organism. The probability that the diabetic organism oxidizes as much glucose as the non-diabetic, renders it unlikely that this hypothesis is correct. We have, in this laboratory, obtained no evidence of an increase in lactacidogen content of the muscle of rabbits after the administration of massive doses of glucose and insulin (Eadie, Macleod and Orr, 1925).

SUMMARY

The administration of glucose to fasting depancreatized dogs that have been deprived of insulin for several days, does not cause similar changes in the phosphorus content of three-hour urine fractions, to those observed in normal animals. The theoretical significance of this observation is outlined.

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STUDIES ON THE INNERVATION OF SMOOTH MUSCLE

IV. Functional Relations between the Lower End of the Esophagus and Stomach of the Cat

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It is the purpose of this paper to give an account of certain observations on functional relations between the lower end of the esophagus and stomach of the cat, made incidentally in an investigation on the effects of vagus stimulation on these structures. The experimental method has been described previously (1), the viscera in no case being exposed to direct observation.

Results. A periodicity in the motor activities of the lower end of the gullet and stomach is often observable in cats anesthetized with chloralose or decerebrated, the extrinsic nerves being intact (2), (3). Phases of rest, in which the musculature relaxes, alternate with phases of increased tonus and peristaltic activity. Relaxation begins in both parts of the canal about the same time, but a marked difference is seen in the promptness of resumption of activity, as illustrated in figure 1. One or more strong contractions appear in the esophageal record, while the stomach is yet quite completely relaxed. Several seconds later, however, the tonus of the stomach rises and evidence of peristaltic waves appears in its tracing. The state of activity then continues until the next rest phase occurs, when the cycle is repeated (fig. 1). This periodicity occurred, furthermore, when no rise of the larynx, indicating deglutition, was seen, though a more delicate method might have revealed such a movement. Much the same alternation takes place, however, when the relaxation is plainly caused by an act of swallowing. In one experiment, confirmatory evidence was obtained of the prediction of Cannon and Lieb (4), that the cardiac end of the stomach is well relaxed when the peristaltic wave set up by deglutition passes over the lower end of the esophagus.

Deflation of the esophageal balloon, rather early in the course of the experiment from which the figures are taken, led to a disappearance of periodicity in the activity of the stomach. The viscus passed into an evenly maintained and well-pronounced state of tonus, and its peristalsis progressed without interruption. Inflation of the balloon, under these cir-

cumstances, caused a marked relaxation of the stomach, as shown in figure 2, and this was accompanied by peristalsis of the lower end of the

Fig. 1

Fig. 2

Fig. 1. Illustrating the periodicity in the activity of the lower end of the esophagus and stomach, and the quicker resumption of activity by the former. The sharp peaks marked x on the stomach tracing are the result of contractions of the abdominal muscles. Cat. Decerebrated by pithing. Extrinsic nerves intact. The two vertical lines on the left represent simultaneous ordinates. In all three figures, the stomach record is above the esophageal; the vertical distance between the short horizontal lines measures the indicated volume change in the stomach balloon; time is in 5-second intervals, and the tracings read from left to right.

Fig. 2. Relaxation of the stomach in response to sudden inflation of the esophageal balloon. Cat. Decerebrated by pithing. Extrinsic nerves intact.

gullet. The preceding condition of tonus and peristaltic activity of the stomach was regained promptly, however, with a succeeding collapse of the esophageal balloon (fig. 2). In case the balloon was left inflated for a

period of several minutes, the interrelation of periodicity reappeared. Rather late in the experiment, however, after one vagus had been cut, the lower end of the esophagus became quite inactive, and periodicity in the activity of the stomach disappeared, though the esophageal balloon remained distended (fig. 3).

This receptive relaxation of the stomach, on local distention of the lower end of the gullet, was observed in both decerebrate cats and those anes-

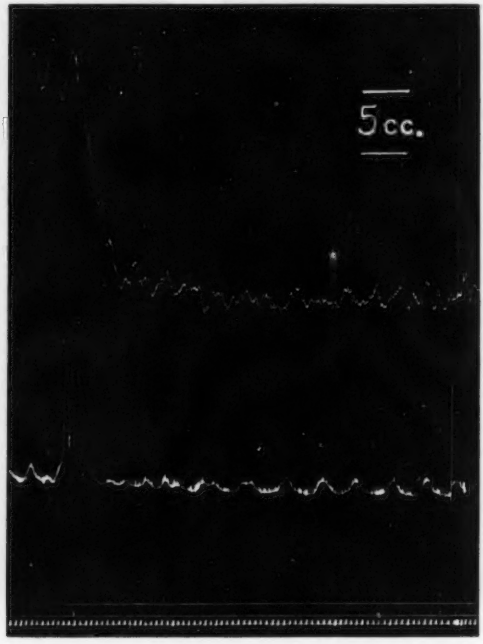

Fig. 3. Effect of ligating right vagus, the time of ligation being indicated by the upstroke on the middle signal line. Left vagus cut previously. Cat. Decerebrated by pithing. Cord pithed (see text).

thetized with chloralose. Severing one vagus nerve does not modify the reaction to a marked degree, but bilateral vagotomy abolishes it entirely. In one experiment, sectioning the vagi resulted in a pronounced and maintained increase in gastric tonus, but the receptive relaxation of the stomach was abolished nevertheless.

Vagotomy may have a variety of effects on the stomach. In one case, with chloralose anesthesia, cutting the left vagus caused an immediate contraction of the stomach, leading to the expulsion of 29 cc. of air from its

balloon, and to an increase in intragastric pressure of considerably more than 10 cm. of water. The entire effect was over, however, in the course of 30 seconds. Sectioning the right nerve soon afterward had a similar but much weaker action, the contraction level returning to that preceding vagotomy. Bilateral vagus section, furthermore, may result in a pronounced increase in gastric tonus, persisting throughout an experiment lasting for hours. The elevation is usually gradual, and the reaction is apparently favored by a condition of inactivity and low tonus (3). If the activity of the stomach is pronounced, however, the result is usually quite different. When the first nerve is cut, an immediate relaxation takes place, but the original level is restored in the course of a few minutes. On sectioning the second vagus, however, a more permanent fall in tonus occurs, and this low level persists during an experiment lasting for hours. The reaction is similar to that shown in figure 3 for ligation of the second vagus. This figure indicates also that the magnitude of the peristaltic waves is diminished. Before taking the tracing represented in figure 3, an attempt had been made to pith the medulla spinalis from the sacral region to the level of the second thoracic vertebra, but the completeness of the pithing was not determined by autopsy.

The effect of vagus section on the lower end of the gullet, in animals anesthetized with chloralose, depends on its condition at the time. If peristalsis is in progress and the musculature is in a condition of considerable tonus, bilateral vagotomy usually has little immediate effect. In the course of a few to forty-five minutes, however, its activity gradually ceases. Periods of rest are interrupted by periods of activity, the former becoming steadily longer. It is questionable, however, whether this gradual cessation can be attributed to cutting the vagi. If the structure is quiescent, on the other hand, sectioning either vagus has a more or less pronounced motor effect. This frequently persists for several minutes, but in the decerebrate cat, the reaction to ligation of the nerve may last only a few seconds (fig. 3). Judging from the graphic record, the reaction consists of active peristalsis together with an increase in tonus. It is apparently always temporary, however, even though a lasting increase in gastric tonus as a result of cutting the nerves, may occur at the same time. Carlson, Boyd and Pearcy (3) have found, in addition, that section of the vagi may cause temporary inhibition of the cardia, when it is in a condition of strong tonus, and that occasionally this procedure may result in spasm of the cardia lasting for several hours.

Discussion. The results described above indicate that the vagus nerves are involved in coördinating the movements and tonus of the lower end of the esophagus and stomach in a purposive manner. Contraction of the former before the latter would tend to force an obstacle in the lower end of the gullet into the relaxed viscus below. Somewhat similar chrono-

logical relations have been described for the pars pylorica of the stomach and the duodenum by Joseph and Meltzer (5), for the pars pylorica, pylorus and duodenum of the dog by Wheelon and Thomas (6) and for the ileum and colon of the cat by Lyman (7). The continuance of contraction of the lower end of the esophagus throughout the period of activity of the stomach probably has for its purpose the prevention of escape of stomach contents (2). It appears from the effects of inflation of the balloon at the lower end of the gullet, furthermore, that the arrival of a bolus of food in this region would be sufficient to relax the stomach reflexly for its reception.

The failure of this receptive relaxation to occur after bilateral vagotomy, even when this procedure results in an increase in gastric tonus, might be considered evidence that the myenteric reflex does not ordinarily function between the parts of the canal involved. It is of interest in this connection that Cannon (8) found it necessary to wait three or four days after cutting the vagi to obtain the best evidence of the functioning of the reflex in preparations from these structures. It might be assumed, therefore, that the failure of the receptive relaxation to take place immediately after bilateral vagotomy is the result of a condition of myenteric plexus "shock." As a counter assumption, it might be suggested that normally unused nervous pathways in the myenteric plexus would become open, in the course of a few days, to the passage of impulses.

The brevity of the effect regularly produced on the lower end of the gullet, and at times also on the stomach, by vagus section, indicates that the mechanical stimulation of cutting lasts at most for only a few minutes. The more permanent effects on the stomach of bilateral vagotomy, therefore, are evidence that these nerves may be involved in tonic excitatory or inhibitory reflexes on the gastric musculature. Whether the splanchnic nerves also are concerned in these or in the receptive relaxation was not determined. That the latter is not conditioned by an output of adrenin, however, is indicated by the brevity of the latent period of the reaction (9), (10) (fig. 2).

SUMMARY AND CONCLUSIONS

1. A purposive functional interrelationship, the extrinsic nerves being intact, is exhibited by the lower end of the esophagus and stomach of the cat. Their activity is roughly synchronously periodic, but the former resumes its contractions earlier than the latter. Inflation of the esophageal balloon, furthermore, causes relaxation of the stomach, accompanied by peristalsis of the lower end of the gullet.

2. Bilateral vagotomy abolishes this receptive relaxation. It is suggested, therefore, that the myenteric reflex may not function, under ordinary conditions, between the parts of the alimentary canal involved.

3. Bilateral vagotomy produces *a*, in the stomach, a temporary motor

reaction or a maintained increase or decrease in tonus, and *b*, in the lower end of the gullet, usually a temporary motor effect, though there may be little change if this structure is in a state of activity.

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ADRENAL SECRETION PRODUCED BY ASPHYXIA

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Asphyxia produces a marked epinephrin discharge. This fact reported by several authors has been denied by others. Our present observations confirm the existence and give some insight into the cause of this phenomenon.

REVIEW OF LITERATURE. Several methods have been used to study adrenal secretion during asphyxia.

Chromaphil reaction and epinephrin content. Asphyxia produced by closing the trachea or breathing carbon monoxide (illuminating gas) diminishes the intensity of the chromaphil reaction and the epinephrin content of the adrenals in dogs, rabbits, cocks and monkeys (Kahn, 1912; Borberg, 1913). The results were obtained by comparing one adrenal taken out before asphyxia with the other taken out after asphyxia.

Kodama, working with cats and dogs asphyxiated by closing the trachea, found a smaller epinephrin content in the adrenals than is usually found in normal animals.

Direct estimation of adrenal secretion. Some investigators have simply measured the epinephrin concentration of adrenal blood. Cannon and Hoskins (1911) introduced a catheter through the femoral vein into the *vena cava* up to the level of the lumbo-adrenal veins. Blood so obtained after asphyxia inhibited the contractions of the isolated intestine of rabbits, while blood from the femoral vein remained inactive. This effect is due to adrenal secretion as it is not observed once the adrenals have been taken out, unless asphyxia is extreme.

Czubalski (1913) also found that the defibrinated blood of dogs drawn after asphyxia inhibits the contractions and relaxes the isolated intestine.

Gley and Quinquaud (1917) measuring the blood-pressure-raising effect reported an increase of two to three times the epinephrin concentration of adrenal blood in asphyxiated dogs, but attach no importance to this fact as a cause of the rise in blood pressure produced by asphyxia.

Volhard and Hülse (1923) studied three curarized dogs and could not find epinephrin in citrated blood (Trendelenburg's surviving-frog method) taken from the femoral vein or the heart, even after asphyxia. Curarization and the use of blood from the general circulation suffice to explain these negative results.

Stewart and Rogoff have very justly criticized all these experiments on the ground that only epinephrin concentration was measured and no account taken of the total output. Simple modifications of circulatory conditions may produce great variations in the epinephrin concentration. To ascertain a true increase in adrenal secretion it is necessary to measure the amount of epinephrin secreted per minute. This they have done and Kodama has repeated their experiments. Stewart and Rogoff's numerous papers were published between 1916 and 1920. They used anesthetized and decerebrated (1917) cats and dogs. The epinephrin output was estimated in blood collected in a "cava pocket" and the effect of this blood was tested on a rabbit's isolated intestine and uterus (1917). They found no increased epinephrin output during or after asphyxia. These results they confirmed by the use of the denervated iris (1916) and blood-pressure curves (1916) which served as internal tests in the asphyxiated animals.

Kodama (1924) followed the same technique as Stewart and Rogoff but his results were absolutely contradictory to those obtained by them. In 11 cats and 6 dogs he found that asphyxia produced an increased epinephrin discharge. At the end of the experiment the epinephrin content of the adrenals was estimated and found to be slightly smaller than that of normal dogs; in cats no such alteration was demonstrated.

Blood pressure as an internal test. Anrep (1912) observed that asphyxia produced a smaller rise in blood pressure when the adrenals were excluded from the circulation.

Czubalski (1913) reported that asphyxia causes a rise of blood pressure in 3 to 4 minutes even after section of the medulla, spinal cord, splanchnic nerves and vagi. Sometimes this rise was considerable, in one case from 34 to 100 mm. Hg. Adrenalectomy suppresses the increase of blood pressure. He supposed that the excess CO_2 prevented the normal destruction of epinephrin secreted into the blood stream and allowed it to accumulate until a sufficient quantity was present to produce effects observed.

Stewart and Rogoff (1916) collected adrenal blood in a "cava pocket" during a given time and measured the blood-pressure-raising effect in the same animal before and after asphyxia (closure of the trachea) observing identical increases in both cases.

Gley and Quinquaud (1917–18–21–23) maintain that the rise of blood pressure produced by asphyxia depends on the integrity of the central nervous system. Adrenalectomy, or ligature of the adrenal veins, section of both major splanchnics, evisceration (removal of stomach, intestine, pancreas, liver, spleen and kidneys) do not modify asphyxial vasoconstriction. In some cases a less marked increase was observed after tying the adrenals but this fact was not considered proof of an epinephrin effect. Asphyxial vasoconstriction was seen in the blood vessels of the lungs, abdominal viscera and musculo-cutaneous structures.

Denervated limb. Anrep (1912) showed that during asphyxia (80 seconds after commencement in the record published) produced by closing the trachea, there is a constriction of the denervated limb (dog, apparently) without any previous dilatation. If the adrenals have been tied off, or the splanchnics cut, no constriction is present.

Itami (1912) also observed constriction of the denervated limbs of dogs breathing 10 to 12 per cent mixtures of CO_2. The volume of the limbs followed the blood-pressure changes passively when the spinal cord was destroyed from the second cervical segment downwards. He suspected an adrenal factor in asphyxial vasoconstriction.

Pearlman and Vincent (1919) were not able to confirm these results. This might be owing to the fact that they used ether, curare and morphia as anesthetics and so diminished the reactions of the adrenals and the denervated limbs. As will be seen later, we have obtained results similar to those reported by Anrep.

Suprareno-jugular anastomosis. In 1923 Tournade and Chabrol described a method of studying adrenal secretion consisting in the anastomosis of the lumbo-adrenal vein of one dog (donor) with the jugular vein of another (recipient) so that all blood leaving the first animal's adrenal was emptied into the circulation of the receptor. The donor's remaining adrenal was taken out. Asphyxia in the donor produced a rise in blood pressure and a slowing of the heart beat in both animals due to a nervous mechanism in the donor and to a humoral influence in the recipient. Section of the major and minor splanchnic nerves suppresses these results. When only the major splanchnics are severed an adrenal discharge may, in some cases, be observed.

In other experiments the kidney of one dog A, was supplied with blood by a second dog, B. Asphyxia in A produced constriction of its own kidney through the vasomotor nerves that had been left intact. Asphyxia in B also produced constriction of A's kidney through an adrenal discharge, no constriction being present when B had been previously adrenalectomized.

The denervated iris as an internal test. Budge in 1855 and later Anderson (1903) described a dilatation of the denervated iris during asphyxia, the so-called "paradoxical reaction." Elliot (1912) ascribed this phenomenon to an adrenal influence. Moderate asphyxia produced a dilatation of the iris denervated by extirpation of the superior cervical ganglion. After adrenalectomy no dilatation was observed unless asphyxia was extreme.

Kellaway (1915) confirmed these results and found that it was sufficient to sever both splanchnics to prevent pupillary dilatation during moderate asphyxia.

Stewart and Rogoff (1916) did not confirm these conclusions with their "cava-pocket" method. Adrenal blood was collected during a given time

and then thrown into the circulation; the denervated iris dilated to the same extent before and after asphyxia. Cats with adrenal secretion suppressed (by extirpation of one and denervation of the other adrenal) showed a dilatation of the iris equal to that of normal animals (1916). Later (1920) they observed that Kellaway's experiments were not valid as proof of an increased adrenal secretion, an accelerated circulation might have brought in a given time a larger quantity of epinephrin to the denervated iris without the existence of a true increase in secretion.

Dilatation of the denervated iris is due, according to Kellaway (1919), to an adrenal discharge produced by a diminished oxygen supply; the effective decrease being 7 to 9 per cent, while the accumulation of CO_2 will give no result even when it reaches a concentration of 15 per cent. Section of the splanchnics, adrenalectomy or both operations done simultaneously suppresses the dilatation. Extreme asphyxia produces a certain degree of dilatation that may be explained by an epinephrin discharge of the accessory chromaphil tissue. It is also possible to prevent the appearance of such a dilatation by destroying the adrenal medulla by means of tubes of radium emanation placed within the gland.

Hartman and Hartman (1923) obtained dilatation of the denervated iris after 40 seconds of asphyxia in cats with only one adrenal, although the remaining gland had its medulla destroyed by cauterization. This result was explained by attributing to the cortex the capacity to secrete epinephrin. Hartman, McCordock and Loder (1923) observed a prolonged dilatation of the denervated iris if the preparation had been made by extirpating both the superior cervical ganglion and the ciliary ganglion. In this condition the pupil remained dilated 4 minutes after asphyxia, and in some cases as long as 30 minutes. The dilatation disappears if the adrenal veins are tied, and reappears once the circulation is restored.

The denervated heart as an internal test. The increase in heart rate produced by slight asphyxia is suppressed when the adrenal veins are ligatured in an unanesthetized dog (Gasser and Meek, 1914). Asphyxia accelerates the rate of the denervated heart owing to an increased adrenal secretion (Cannon, 1919). Stewart and Rogoff (1920) deny all importance to the adrenal discharge in this case, arguing that the smaller increases in heart rate can be observed without any alterations in the adrenal secretion, owing to low blood pressure produced by the trauma of the experiment.

Cannon and Carrasco-Formiguera (1922) confirmed Cannon's previous work. Asphyxia during 45 seconds increases the heart rate in a cat with denervated heart and liver. When the adrenal blood does not enter the circulation (closure of the *vena cava*) asphyxia does not produce an increase in heart rate, but does so again if the adrenal circulation is restored.

Searles (1923) observed in fasting dogs an increase in rate of the denervated heart after asphyxia, which did not occur if the lumbo-adrenal veins were tied and reappeared once the ligatures were removed.

Hyperglycemia as an internal test. Starkenstein (1912) maintained that hyperglycemia produced by asphyxia does not occur in the adrenalectomized rabbit. Stewart and Rogoff (1917–18–20) deny any adrenal factor in this increase of blood sugar. Asphyxia produces the same degree of hyperglycemia in cats with one adrenal extirpated and the other denervated as it does in normal animals (1917). Adrenalectomized animals have a normal hyperglycemia after asphyxia if time is given them to recover from the operation (1918). Kellaway (1919) observed hyperglycemia in adrenalectomized cats after asphyxia. He concludes that other factors besides the adrenals influence the blood sugar, but remarks that quantities of epinephrin too small to cause pupillary dilatation produce a definite increase of the blood sugar. Anoxemia is the primary cause of these results.

Brain temperature. Asphyxia is accompanied by an increase of temperature in the brain due to adrenal discharge as it is not observed once the adrenals have been taken out. (Crile, Rowland and Wallace, 1923.)

ORIGINAL EXPERIMENTS. A marked adrenal discharge is produced by asphyxia. We have observed it in the following circumstances: 1, closure of the trachea; 2, injection of curare or cobra venom; 3, potassium cyanide administration, and 4, breathing of several gas mixtures.

A. CLOSURE OF THE TRACHEA: All experiments were performed on dogs anesthetized with chloralose (0.10 gram per kgm., intravenously injected), with both vagi cut and under artificial respiration. As tests we used the denervated limb and the suprareno-jugular anastomosis. In some cases quantitative estimations were made.

The denervated limb as internal test: Two experiments performed confirmed Anrep's previous work. Blood-pressure and hind-leg-volume curves (Dale and Richard's plethysmograph) were taken. The trachea was closed by means of a hemostat for 4 and 6 minutes. Ninety seconds and 150 seconds after the commencement of asphyxia an intense constriction of the leg was recorded lasting 3 and 5 minutes. The adrenals were taken out in one animal and both major and minor splanchnics severed in the other. A new period of asphyxia lasting 5 minutes did not produce constriction of the leg and the increase in blood pressure was not as great as in the first case.

Suprareno-jugular anastomosis: This excellent method of Tournade and Chabrol is free from all criticism as a medium to study asphyxial adrenal secretion. The central end of the left lumbo-adrenal vein of a dog (donor) was ligatured, the peripheral end was united to the jugular vein of a second dog (recipient) by means of a venous segment. All the adrenal blood from the left gland of the first dog passes into the circulation of the second,

whose weight was about 50 per cent less. The recipient's heart was denervated. Both animals were under artificial respiration.

In four experiments the donor's trachea was closed from 4 to 6 minutes. Two to five minutes after the start an intense adrenal discharge was registered by a marked rise in blood pressure and increased heart rate in the recipient. The blood pressure rose sharply in the donor before it did so in the recipient and remained high for 3½ to 6 minutes. The heart rate increased and remained rapid during this same lapse of time but gradually came down to the previous rate.

In one case when the respiration was reëstablished a rise in blood pressure was observed in both dogs.

TABLE 1

DATE	WEIGHT		ASPHYXIA DURATION	TIME OF RESPONSE OF RECIPIENT	BLOOD PRESSURE RISE		BLOOD PRESSURE RISE DURATION		INCREASE IN HEART RATE OF RECIPIENT	DURATION OF INCREASE IN HEART RATE
	Donor	Recipient			Donor	Recipient	Donor	Recipient		
	kgm.	*kgm.*	*minutes*	*minutes*	*mm.Hg*	*mm.Hg*	*minutes*	*minutes*		*minutes*
5/18/1925	18	8	6	5	114	264	9	4½	90	4
5/20/1925	17	9.5	5	4	44	84		7	24	5
5/22/1925	18	9	4	2	110	90	7	6	26	6
5/26/1925	15	10	4	2¼	100	160	8½	5	52	3½

Quantitative measurements of adrenal discharge: The adrenal discharge produced by asphyxia is a large one and lasts longer than that produced by stimulating the splanchnics or the bulb. In some cases the secretion amounts to 0.05 mgm. or more per minute. This quantity is sufficient to produce hyperglycemia, inhibit intestinal movements and diminish the volume of the kidney or the spleen.

Three experiments were performed to measure the amount of epinephrin secreted. The adrenal blood of the donor was collected every minute or every two minutes in tubes with 0.05 gm. of sodium citrate in 1 cc. normal saline. This blood was injected as quickly as possible into the jugular vein of dogs (two animals used in each experiment) whose blood pressure and heart rate were then recorded. At the beginning and end of the experiment tests were made with adrenalin which had been previously titrated by the Cannon, Folin and Denis method, and the increase in blood pressure and heart rate measured.

1st experiment. Donor's weight 20.5 kgm.; the left adrenal weighed 1.52 grams. Asphyxia was produced by closing the trachea for six minutes. The adrenal blood

was tested as described above on two dogs weighing 7 and 8.5 kgm. The following results were obtained:

	BLOOD COLLECTED	EPINEPHRIN CONTENT
	cc.	*mgm.*
1 minute before asphyxia	7.2	<0.001
Asphyxia: Duration in minutes:		
1	6.0	<0.001
2	14.0	<0.001
3	13.0	0.005
4	12.0	0.025
5	7.0	0.045
6	7.5	0.030
7	6.0	0.020
8	3.0	0.002

2nd experiment. Donor's weight 16 kgm.; left adrenal 1.20 grams. Test dogs weighed 9 kgm. each. Asphyxia by closing trachea lasted six minutes.

	BLOOD COLLECTED	EPINEPHRIN CONTENT
	cc.	*mgm.*
2 minutes before asphyxia	4	traces
Asphyxia: Duration in minutes:		
1-2	4	0.003
3-4	10	0.004
5-6	6	0.020

Fig. 1. 5/26/1925. Blood pressure of recipient (10 kgm.) upper curve. Blood pressure of donor (15 kgm.) lower curve. Between *1* and *2* donor's trachea closed.

Fig. 2. 5/22/1925. Upper curve—Blood pressure of recipient. Lower curve—Blood pressure of donor. Between *1* and *2* donor's trachea closed.

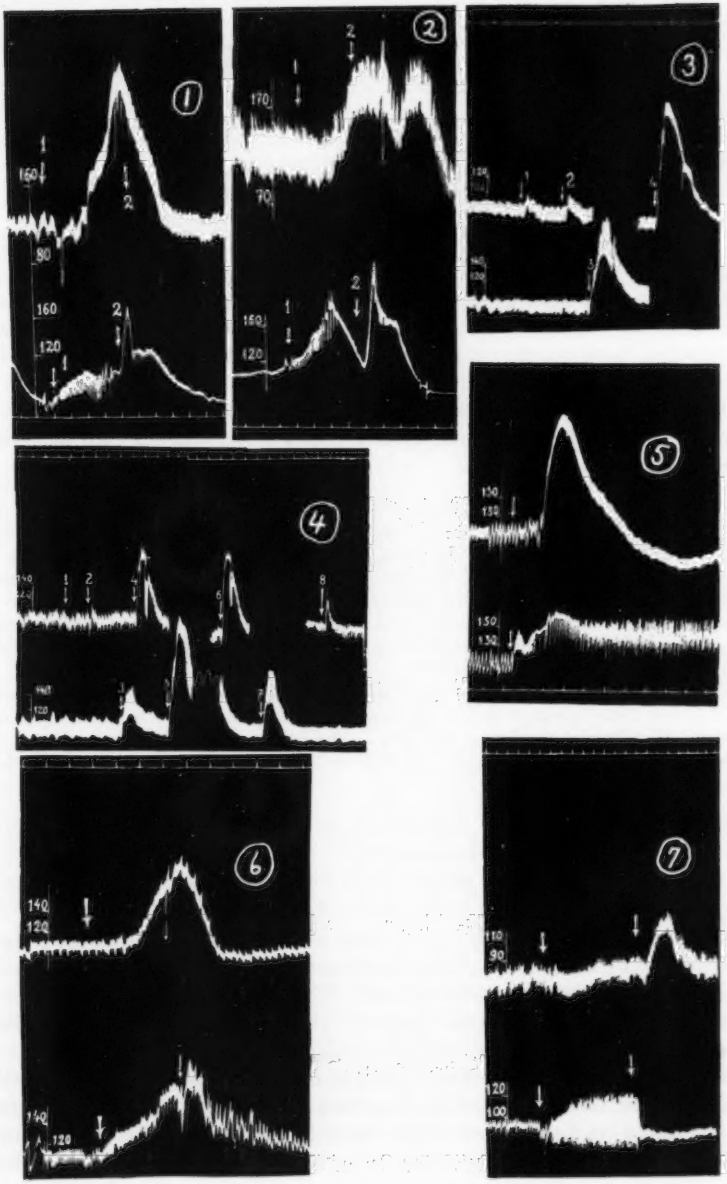
Fig. 3. 6/9/1925. Estimation of amount of epinephrin discharged. Effect of adrenal blood on blood pressure of test animals. *1.* Two minutes before asphyxia. *2.* During 1st and 2d minutes of asphyxia. *3.* During 3rd and 4th minutes. *4.* During 5th and 6th minutes.

Fig. 4. 6/10/1925. Estimation of amount of epinephrin discharged. *1* to *8:* Effects on blood pressure of adrenal blood collected every minute after asphyxia, injected alternately into two test animals.

Fig. 5. 6/30/1925. Upper curve—Blood pressure of the recipient (29 kgm.). Lower curve—Blood pressure of the donor (30 kgm.). Time in minutes—30 mgm. potassium cyanide intravenously to the donor.

Fig. 6. 8/4/1925. Time in minutes. Upper curve—Blood pressure of recipient (9.5 kgm.). Lower curve—Blood pressure of donor (15 kgm.). Between arrows donor breathed a nitrogen atmosphere with only 5.7 per cent oxygen.

Fig. 7. 8/10/1925. Time in minutes. Upper curve—Blood pressure of recipient (12 kgm.). Lower curve—Blood pressure of donor (14 kgm.). Between the arrows the donor breathed air with 16.4 per cent CO_2. Rise of blood pressure in recipient when pure air is restored.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7
110
90
120
100

3rd experiment. Donor's weight 20.5 kgm.; left adrenal 1.55 grams. Test dogs weighed 7.5 and 8 kgm. Asphyxia lasted six minutes.

	BLOOD COLLECTED	EPINEPHRIN CONTENT
	cc.	*mgm*
2 minutes before asphyxia	13	0.0007
Asphyxia: Duration in minutes:		
1-2	12	0.0010
3-4	22	0 0300
5-6	11	0.0650

As can be seen in the protocols, the maximum discharge occurs between 5 and 6 minutes after asphyxia is begun. An increased blood output is present 3 to 4 minutes after commencement of asphyxia. There is no parallelism between the quantity of epinephrin discharged and that of blood circulating through the gland.

B. CURARE AND COBRA VENOM: Curare and cobra venom produce asphyxia by paralysis of the respiratory muscles and therefore an adrenal discharge.

Experiment of 5/27/1925. Donor's weight 17.5 kgm. Recipient's weight 9 kgm. One milligram cobra venom intravenously injected in donor. Artificial respiration was stopped 17 minutes later, asphyxia was complete in a few minutes. Blood pressure rose 36 mm. Hg. The recipient showed a sharp rise of blood pressure (90 mm. Hg) 2½ minutes after asphyxia began and the heart rate increased 20 beats per minute. Three and a half minutes after artificial respiration was stopped it was again started, the blood pressure returned to its original level in 4½ minutes and the heart rate fell to normal in 4 minutes.

Experiment of 6/7/1925. The same technique as in the experiment just recorded, but donor injected with curare instead of cobra venom. The donor's blood pressure rose 50 mm. Hg during asphyxia. The recipient had a sharp rise of blood pressure 3½ minutes after asphyxia started and an increase in pulse rate of 32 beats per minute.

C. POTASSIUM CYANIDE: A dog (donor) weighing 31 kgm. receives 30 mgm. potassium cyanide intravenously. The blood pressure of the recipient (29 kgm.) rose 1½ minute later from 120 mm. Hg to 234 mm. Hg and had an increase in heart rate of 32 beats per minute beginning 2 minutes after the injection and lasting for more than 4 minutes. The donor had only a slight rise of blood pressure, owing perhaps to a diminished sensitiveness of the vasomotor system. Evans has reported that cyanides diminish the blood-pressure response to adrenaline and splanchnic stimulation.

D. BREATHING GAS MIXTURES POOR IN OXYGEN OR RICH IN CO_2. Both animals were anesthetized with chloralose. The donor, whose vagi were intact, had a tracheal cannula with two valves inserted. The inspiratory valve communicated with the open air or with a rubber bag containing the

gas mixtures studied, nitrogen and oxygen or air and CO_2 mixtures (Doctor Artundo checked their composition by means of Haldane's gas-analysis apparatus). The anastomosis was performed; the recipient's blood pressure and rate of denervated heart were recorded. After a few minutes of observation during which the donor breathed room air the inspiratory valve was connected with the gas bag until the content (80 liters) had been used up. Table 2 summarizes the results obtained. Oxygen lack is the efficient cause of the discharge; carbon dioxide apparently plays no part.

When the mixtures were breathed during 10 minutes epinephrin was discharged with O_2 concentrations of 5.7, 5.9, 6.3 and 8.7 per cent. No

TABLE 2

DATE	WEIGHT		GAS MIXTURE BREATHED BY DONOR		TIME	BLOOD PRESSURE RISE		INCREASE IN HEART RATE OF RECIPIENT	TIME RESPONSE STARTED IN RECIPIENT	OBSERVATIONS
	Donor	Recipient	O_2 per cent	Liters		Donor	Recipient			
	kgm.	*kgm.*			*minutes*	*mm. Hg*	*mm. Hg*		*minutes*	
8/ 3/1925	22	8	17		8	0	0	0	0	
8/ 4/1925	15.5	9.5	15	64	10	0	0	0	0	
8/ 4/1925	15.5	9.5	11	40	4½	20	10	0	2	
8/11/1925	23	13	9.3	80	7	0	0	0	0	
8/10/1925	14	12	8.7	81	10	16	0	2	2	
8/ 8/1925	17.5	10	6.3	34	7	10	10	2		Breathing gas mixture
						80	40	14		Pure air just started
8/ 7/1925	17	9	5.9	68	10	30	12	6	1¾	
8/11/1925	15	9.5	5.7	27	3½	90	80	62	2	
			CO_2 per cent							
8/ 7/1925	17	9	4.1	63	15	0	0	0	0	
8/ 8/1925	17.5	10	7	75	8	6	0	0	0	
8/10/1925	14	12	16.4	80	8	30	0	0	0	Breathing mixture
							50	14		Pure air just started
8/11/1925	23	13	28	80	3⅔	−30 +40	0	4		

discharge was recorded with O_2 concentration above 11 per cent. In one experiment an O_2 concentration of 11 per cent produced a slight discharge. When adrenal secretion increased, the blood pressure rose in the donor and gradually rose in the recipient. One case (5.7 per cent O_2) showed a sharp increase in the blood pressure of the recipient 2 minutes after the commencement of asphyxia. When the donor again breathed pure air the blood pressure gradually fell to the previous level in both dogs.

In one experiment (6.3 per cent O_2) when the breathing of pure air was re-established a sharp rise of blood pressure was registered in both dogs accompanied by an increased heart rate in the recipient.

Air rich in CO_2 produced deep breathing and slowed the heart rate in the donor. The recipient's blood pressure never rose and, in one case (16.4 per cent CO_2), there was a fall, followed by a rise when pure air was again given to the donor. The pulse rate was slower, 2 beats per minute with 7.7 per cent CO_2 and 6 beats per minute with 16.4 per cent CO_2. When 28 per cent CO_2 was breathed, a rise of 2 beats per minute was followed by a fall of 2 beats.

In conclusion it can be stated that a lowered oxygen pressure increases adrenal secretion. Increased CO_2 tension does not influence it to any great extent; it seems to diminish it slightly, though in one experiment (28 per

TABLE 3

DATE	WEIGHT		ASPHYXIA DURATION	BLOOD PRESSURE VARIATION		INCREASE IN HEART RATE RECIPIENT	OPERATION PERFORMED
	Donor	Recipient		Donor	Recipient		
	kgm.	*kgm.*	*minutes*	*mm.Hg*	*mm.Hg*		
6/ 1/1925	15.5	12.5	3	0	0	0	Major splanchnic cut
6/ 1/1925	15.5	12.5	4	0	0	0	Major splanchnic cut
6/ 2/1925	24	5	4	0	0	0	Major splanchnic cut
6/25/1925	19	11.5	5	30	0	0	Major splanchnic cut
6/ 3/1925	18	10	4	30	0	0	Major and minor splanchnic cut
6/ 5/1925	23	9.3	5	74	0	0	Major and minor splanchnic cut
7/20/1925	23.5	11	6	0	0	0	Spinal cord cut just below medulla

cent CO_2) perhaps a small increase may have been present. A study of the effect of CO_2 tension with a more sensitive method might possibly give clearer results.

Rôle played by the nervous system. Our experiments confirm Tournade and Chabrol's results. Asphyxia does not produce an increased adrenal secretion when the major splanchnics are cut in the donor (two experiments). Sometimes, as Tournade and Chabrol have remarked it is necessary to cut the minor splanchnic to prevent adrenal discharge. Complete denervation of the adrenal (see previous paper on nicotin, etc.) efficiently prevents all adrenal secretion. Section of the spinal cord just below the medulla has the same effect, though Czubalski's paper seems to indicate that in some cases asphyxia may stimulate adrenal centers. (Table 3.)

SUMMARY

1. Closure of the trachea produced a marked adrenal discharge in 2 to 5 minutes reaching the maximal point in 5 to 6 minutes. It can be as much as 0.045 mgm. per minute.

2. This discharge was registered by a recipient dog showing *a*, rise of blood pressure; *b*, increased heart rate; *c*, constriction of the denervated limb.

3. Respiratory paralysis produced by curare or cobra venom produces asphyxia and consequently adrenal discharge.

4. Potassium cyanide produces adrenal discharge.

5. Diminished oxygen tension is the efficient cause of the discharge in these cases. CO_2 accumulation has no apparent effect.

6. Asphyxia acts on the central nervous system and the stimulus is transmitted through the splanchnics.

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EFFECT OF NICOTIN, CYSTOSIN, LOBELIN, CONIIN, PIPERIDIN AND QUATERNARY AMMONIAS ON ADRENAL SECRETION

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Nicotin is the type of a group of substances, one of whose characteristic properties is the capacity first to stimulate then to paralyze sympathetic ganglion cells (Langley, 1890, 1896, 1919; Langley and Dickinson, 1889, 1890).

The adrenals receive sympathetic innervation by means of the major splanchnics and accessorily through the minor splanchnics. The adrenal medulla has a common embryological origin with the sympathetic ganglia Epinephrin produces sympathico-mimetic effects. All these facts lend a special interest to the study of the influence of nicotin and drugs of similar properties on adrenal secretion.

PREVIOUS WORK. Mansfeld (1908) seems to have first recorded an increased adrenal secretion after nicotin injections. Unhappily we have not been able to obtain his paper.

Direct estimation of epinephrin secreted. Cannon, Aub and Binger (1912) studied the problem in cats. Blood was collected from the vena cava at the level of the adrenals by means of a catheter introduced into the femoral vein. The epinephrin content of this blood was determined by means of the isolated-intestine method. Injections of 3.5 to 7.5 mgm. nicotin considerably increased the epinephrin concentration in 3 to 5 minutes and maintained it for several minutes (in one experiment for 12 minutes). Adrenalectomy suppresses this effect. Stewart and Rogoff (1919) remarked that this increased concentration might be due to a diminished blood flow through the adrenals brought about by the initial bradycardia caused by nicotin. This objection, though plausible, is not valid, as will be seen later; our present experiments clearly show that Cannon's results were caused by an increased epinephrin secretion.

Steward and Rogoff (1919) by means of the "cava pocket" method made quantitative determinations, measuring the amount of epinephrin secreted in a given time (the isolated intestine and uterus being used as tests). Nicotin, after a few seconds, produces in cats a considerable increase in epinephrin output; 15 and 20 times the normal quantity per minute and,

in some cases, high concentrations such as are never seen under any other experimental conditions. This stimulation lasts only a very short time, from 30 to 60 seconds, and is followed by a prolonged period of depression. The diminished epinephrin secretion is the predominant action of nicotin; it is observed after subcutaneous and intravenous injections and is sometimes so marked that no epinephrin is found in the adrenal blood. Gradually the adrenal secretion is restored to normal conditions. These different phases are crudely parallel to the blood-pressure variations and evidently are due to the effects of nicotin on the ganglion cells of the efferent epinephrin secreting paths. In one experiment strychnin was injected after nicotin and the usual adrenal discharge was not observed. The adrenals retain, according to these authors, their normal stock of epinephrin as no difference was found between the epinephrin content of a denervated adrenal and that of the opposite adrenal whose nerves had been kept intact. Later we shall see that nicotin acts directly on the cells of the adrenal medulla.

Eichholtz (1923) used cats anesthetized with urethane to study the effects of several drugs on the epinephrin content of blood taken from the lumbo-adrenal and renal veins (Trendelenburg's surviving frog method as test). Nicotin increases adrenal secretion as much as 0.0005 to 0.009 mgm. per minute per kilo, even when the splanchnic nerve has been previously severed. Similar results were obtained with hordenin bromethylate, tetramethylammonium chloride (in this case on animals injected with atropin) and neurin. Other bases of quaternary ammonias did not increase epinephrin output; e.g., acetylcholin and tetrahydrobetanaphthylamin.

Stroomann (1925) reports an adrenal discharge in man after nicotin administration (Trendelenburg's method being used as test).

Chromaphil reaction and epinephrin content. Elliott (1912) reported the appearance of symptoms denoting sympathetic activity in cats injected with tetrahydrobetanaphthylamin that portray a state of fright, dilatation of the iris, exophthalmos, erection of hairs, etc. The adrenals of these animals contained very little or no epinephrin unless the splanchnics had been previously cut. As we have already mentioned, Stewart and Rogoff (1919) deny adrenal depletion after nicotin injection because a denervated adrenal contains the same quantity of epinephrin as the gland whose nerves are left intact.

Direct effect on a surviving adrenal. By perfusion of the surviving adrenals of cattle with Ringer's solution, at 38°C. it is possible to obtain a fluid that has the properties of epinephrin. There is not only a washing out of epinephrin already present in the gland, but apparently a new formation of this substance or rather of another very similar to it but more stable and resistant to alkali. Adding nicotin to the perfusing fluid increases the

epinephrin output four to six times, although no vascular effect is recorded (Schkawera and Kusmetzow, 1923; Kudrjawzew, 1924). Epinephrin was estimated in these experiments by means of several tests; its action on the blood vessels of the rabbit's ear, human finger, rabbit's kidney, etc. Nikolaeff (1924) also noted an increased epinephrin output, accompanied by a slight vasoconstriction, when coniin was perfused. The effect lasted one hour or more; after two hours a decrease was observed.

Krichel (quoted from Eichholtz, 1923) ascertains that the surviving adrenal vein of a horse is not sensitive to nicotin nor to the bases of quaternary ammonias.

Blood pressure as an internal test. Nicotin (Wertheimer, 1891; Gley, 1914) and anargyrin (Gley, 1892) produce a considerable increase of blood pressure in dogs whose medulla and spinal cord have been destroyed. Adrenalectomy suppresses this effect almost completely. A slight increase may be observed, especially when anargyrin is used, owing to direct action on the blood vessels or on the sympathetic ganglia. Usually they produce vasoconstriction indirectly, discharging epinephrin. Stewart and Rogoff (1919) maintain that Gley should have tested, by means of epinephrin injections, the vasomotor reactions of their animals before and after adrenalectomy, as this operation may alter their ability to respond. Our present experiments will show the exactness of Gley's conclusions.

Langley (1919) obtained an increase of blood pressure after nicotin injection even when the adrenals had been excluded by ligation, a fact that Stewart and Rogoff confirmed. These authors collected the adrenal blood of cats anesthetized by urethane in a "cava pocket" and studied the effects it produced on the blood pressure. After a pronounced but transitory increase of adrenal secretion, they observed, by this method, a marked and prolonged diminuton of the epinephrin output.

The uterus as an internal test. Dale and Laidlaw (1912a) used this method in an important paper describing the effects of several drugs. They used female cats, whose central nervous system had been destroyed, liver excluded from the circulation, and bowels take out. Contractions of the uterus horn were recorded. Nicotin, cystosin and hordenin methyliodid inhibited movements and relaxed the uterus. The isolated uterus generally reacts to these drugs with contractions. Lodal, a derivative of laudanosin, has similar discordant effects *in vivo* and *in vitro*. Stimulation of the major splanchinics produces a relaxation of the uterus, owing to an epinephrin discharge. Lodal does not relax the uterus after adrenalectomy.

The denervated iris as an internal test. In cats nicotin injections dilate the pupil, retract the nictitating membrane and widen the eye fissure. Painting the superior cervical ganglion with the drug produces the same effects, but since they were observed even when the ganglion had been previously taken out, it was supposed that the drug also acted on a peripheral mechanism (Langley and Dickinson, 1890).

Dale and Laidlaw's (1912b) experiments showed that the effects of nicotin on the eye were due to an adrenal discharge. Dilatation of the iris was observed in eyes 13 days after their denervation, that is to say, when all postganglionic fibers had degenerated. Adrenalectomy and temporary closure of the aorta above the adrenal arteries suppressed the results usually observed after injecting nicotin, cystosin and lobelin except for a slight, transitory dilatation. When circulation was reëstablished in the aorta, the eye effects were observed, their intenseness being somewhat diminished but not totally suppressed if the splanchnics had been severed. These drugs have no action on the enucleated eye of the cat. In cats anesthetized with urethane, dilatation of the pupil is seen even after adrenalectomy and extirpation of the superior cervical ganglion. Nicotin and other drugs of similar properties may produce their effects by three different mechanisms: *A*, stimulation of the superior cervical ganglion; *B*, adrenal discharge, and *C*, peripheral sympathico-mimetic action. These same authors report that cystosin dilates the iris of cats whose superior cervical ganglion has been taken out.

In these experiments the ciliary ganglion had not been removed. Langley (1919) remarks that nicotin might have produced these effects by paralysis of this ganglion. Dale and Laidlaw gave cats curare until the cervical sympathetic did not respond to stimulation; in this condition nicotin produces a constriction followed by a dilatation of the pupil, but larger doses are necessary as if it were necessary to overcome a paralysis of the adrenal secreting fibers produced by curare. In one experiment after adrenalectomy a dose of 10 mgm. of nicotin did not dilate the pupil. In two other animals, one curarized and the other injected with brucin, nicotin dilated the pupil but did not affect the nictitating membrane, owing to an adrenal discharge, not to a direct effect on the nerve endings of the iris, since these were paralyzed. When cats treated with ergotoxin and strychnin are injected with nicotin it produces an accelerated heart rate, exophthalmos and dilatation of the pupil.

Stewart and Rogoff (1919) extirpated the superior cervical ganglion of cats and observed, by their "cava pocket" method, a dilatation of the iris after nicotin injections.

Shimidzu (1924) worked on rabbits in which the superior cervical ganglion had been extirpated on one side and injected them with atropin so as to suppress the ocular motor nerve. Nicotin slowly injected (0.027 mgm. per kilogram per minute) did not dilate the pupil. Larger doses (0.05 mgm.) produce an intense and prolonged dilatation (five experiments). Ligature of the adrenals suppresses this effect. In one experiment the larger dose of nicotin produced an adrenal discharge of 0.005 mgm. epinephrin per kilo per minute, estimated by comparing the dilatation produced by the injection of a known dose of epinephrin. Tetrahydrobetanaph-

thylamin dilates the denervated iris in rabbits with or without adrenals.

Blood-platelets. Nicotin reduces the quantity of blood-platelets to a remarkable extent in one or two hours. Later the normal count is restored. In one experiment an increased white count was found two and one-half hours after the injection. These changes in the blood picture might be due to an increased adrenal secretion followed by a prolonged inhibition (Backman, Edström, Grahs and Hultgren, 1925).

EXPERIMENTS. Our experiments were performed on sixty dogs anesthetized with chloralose (0.10 gram per kilogram intravenously injected), with both vagi always cut in the recipients, and in the donors cut only in the experiments performed after May 19. Artificial respiration was given. Tournade and Chabrol's technique was followed so that all the adrenal blood of a donor's left adrenal passed into the circulation (jugular vein) of a recipient. In some cases the right adrenal was used. The first dog generally weighed about 20 kgm., the second, between one-half and two-thirds that weight.

The donor's blood pressure was recorded by a mercury manometer. Artificial respiration prevented possible asphyxia by apnea caused by large doses of the drugs studied. These were usually injected into the jugular vein, though sometimes the external saphenous vein was used.

The recipient's blood pressure was registered with a mercury manometer, the heart rate with an elastic manometer (Straub). Both vagi were cut in the neck and the stellate ganglia extirpated following Carville and Rochefontaine's technique (1874).

The following tests were used to register the effect of adrenal secretion of the donor in the recipient: blood pressure, denervated heart, glycemia, intestinal contractions, and the volume of different viscera. The denervated iris was not used because it was considered superfluous to recur to such a complex test and the uterus was discarded as Dale and Laidlaw's experiments were considered conclusive in this respect. In a few experiments adrenal blood was collected before and after the drugs were injected and the quantity of epinephrin secreted estimated by means of external tests.

To measure the amount discharged by the recipient once an effect was obtained on the donor, known quantities of epinephrin were injected in the donor every six to ten minutes until an identical effect was obtained.

Simple anastomosis. Immediately after the establishment of the anastomosis a slight and sharp increase in blood pressure may be registered in the recipient in some cases, owing to an accumulation of epinephrin whilst the anastomosis is effected. In cases where the recipient has an initial low blood pressure the anastomosis produces a sharp or gradual rise. After a very short time the blood pressure remains constant in all these

cases and in the great majority of the experiments no changes at all were seen.

A rise of blood pressure in the donor, produced by stimulating the right splanchnic, after extirpating the right adrenal, or by injecting epinephrin (0.01 to 0.10 mgm.) does not increase the blood pressure or accelerate the denervated heart of the recipient (fig. 1). In one experiment, where the adrenal circulation was considerably retarded (0.25 cc. per minute), an injection of epinephrin in the donor slightly raised the blood pressure in the recipient. We attribute this to the fact that during the slow circulation the maximum adrenal concentration had been reached, epinephrin improved the circulation and so enabled a larger amount of epinephrin to be put out by the adrenal. Similar situations have already been studied by Stewart and Rogoff.

Fig. 1. 5/4/1925. Upper curve—blood pressure of donor (16.5 kgm.). Lower curve—blood pressure of recipient (8.0 kgm.). *1.* Intravenous injection of 0.01 mgm. epinephrin in donor. *2.* Intravenous injection of 16.0 mgm. cystosin in donor.

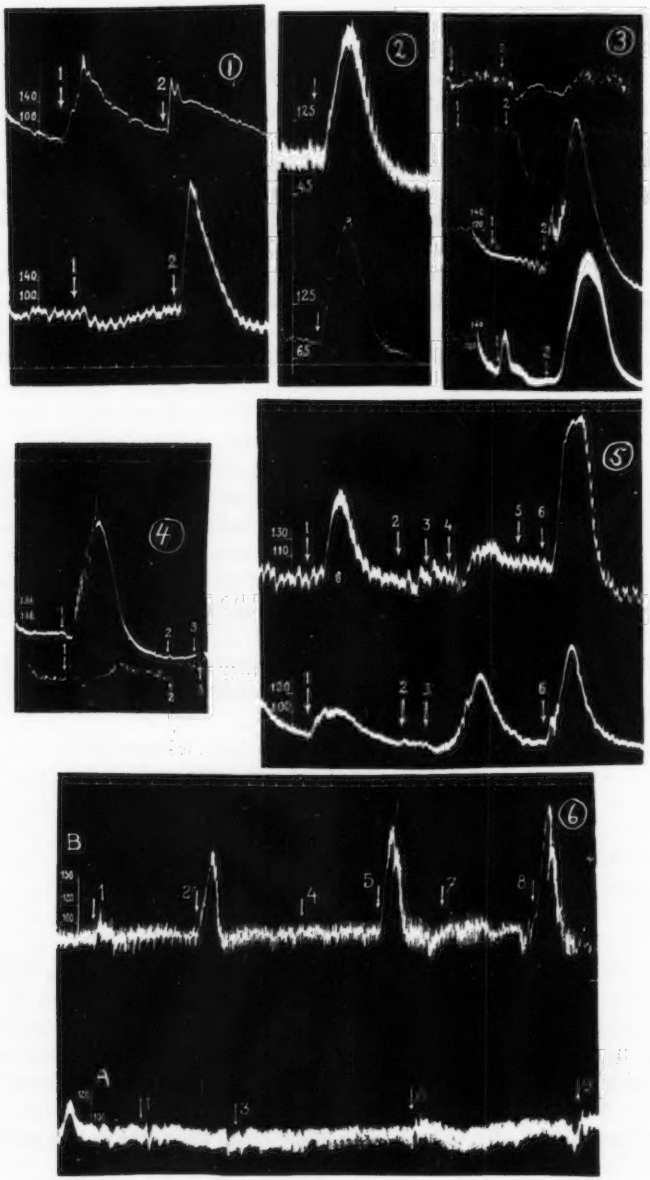
Fig. 2. 7/24/1925. Upper curve—blood pressure of recipient (15.5 kgm.). Lower curve—blood pressure of donor (17.5 kgm.). Time in minutes. Injection 10 mgm. hordenin methyliodid.

Fig. 3. 6/15/1925. Time in minutes. Intestinal movements of recipient (10 kgm.). Volume of spleen of recipient. Blood pressure of recipient. Blood pressure of donor (24 kgm.). *1.* Intravenous injection 0.005 mgm. epinephrin. *2.* Intravenous injection 2 mgm. nicotin.

Fig. 4. 6/22/1925. Dog (18 kgm.) with denervated gut. Upper curve—blood pressure. Lower curve—intestinal movements. *1.* Intravenous injection 1 mgm. nicotin. Adrenals extirpated. *2.* Injection 1 mgm. nicotin. *3.* Injection 2 mgm. nicotin.

Fig. 5. 6/2/1925. Time in minutes. Blood-pressure curve of recipient (8 kgm.). Blood-pressure curve of donor (24 kgm.). *1.* Donor injected intravenously 1 mgm. nicotin. *2.* Transfusion interrupted. *3.* Donor injected intravenously 2 mgm. nicotin. Between 30 and 80 seconds after injection 20 cc. blood drawn from its jugular vein and injected into recipient at *4.* *5.* Transfusion recommenced. *6.* Donor receives 2 mgm. nicotin intravenously.

Fig. 6. 6/6/1925. Blood-pressure of test dogs A and B. *1.* Intravenous injection of 5 cc. "normal" adrenal blood given by donor during 1 minute before having received nicotin. *2.* Intravenous injection of 8 cc. adrenal blood taken from donor during first minute after injecting 2 mgm. nicotin. *3.* Intravenous injection of 15 cc. adrenal blood taken from donor during second minute after injecting 2 mgm. nicotin. *4.* Intravenous injection of 7.5 cc. adrenal blood taken from donor during fifth minute after injecting 2 mgm. nicotin. *5.* Intravenous injection of 7 cc. adrenal blood taken from donor during first minute after injecting 1 mgm. cystosin. *6.* Intravenous injection of 15 cc. adrenal blood taken from donor during second minute after injecting 1 mgm. cystosin. *7.* Intravenous injection of 8.5 cc. adrenal blood taken from donor during fifth minute after injecting 1 mgm. cystosin. *8.* Intravenous injection of 7 cc. adrenal blood taken from donor during first minute after injecting 1 mgm. lobelin. *9.* Intravenous injection of 16 cc. adrenal blood taken from donor during second minute after injecting 1 mgm. lobelin.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A

Effects on the blood pressure and heart rate. Nicotin injections in the donor produce different effects on the recipient according to the doses used; 0.1 mgm. given to a dog weighing 20 kgm. generally has no effect or a very slight one on the donor, 1 mgm. considerably raises the blood pressure in both dogs, and 5, 10, 15 and 18 mgm. doses increase this effect. The donor shows a slowing of the heart beat, sometimes a complete stop of a few seconds, followed by slow beats that afterwards increase in frequency, so that 1 or 2 minutes later a moderate tachycardia is seen, with occasional spurts. When the vagi are cut, the initial slowing is less marked. Blood pressure, of course, drops when the slowing of the heart beat is marked, but rapidly increases whether the heart rate is slow or fast, though in the latter case it goes up higher. Afterwards it gradually returns to the previous level. The period of increase lasts from 3 to 5 minutes.

The recipient shows no effects until after 30 to 60 seconds. Then an increased heart rate and a sharp rise of blood pressure are registered. The maximum heart rate is attained between 30 and 90 seconds after the initial rise and the peak of the blood pressure is always reached within 2 minutes and lasts from 3 to 8 minutes. This rise in blood pressure is more marked than that registered by the donor. Perhaps nicotin diminishes the animal's sensitiveness to adrenalin. The variation of blood pressure lies between 170 and 206 mm. Hg and the increase in heart rate is about 100 beats per minute.

Cystosin and lobelin produce the same results as nicotin under these experimental conditions, but weight for weight they are more active, especially lobelin.

Several quaternary ammonias are also active: tetramethylammonium chloride, bromide and iodide and hordenin iodinmethylate. This last drug gives a marked effect, yet curiously enough an equal dose of hordenin sulphate is inactive. One experiment with tetrapropylammonium iodide gave negative results, as did coniin (5, 10 and 20 mgm.), piperidin (141 mgm.), and tetrahydrobetanaphthylamin (68 mgm.) (table 1).

Effects on intestinal contractions. Nicotin paralyzes intestinal contractions (Bayliss and Starling, 1899). Langley and Magnus (1905) localized the inhibitory effect and ascribed it to a peripheral mechanism because inhibition was observed when all post-ganglionic fibers going to the jejunum had been severed. The isolated intestinal strip is only inhibited by very large doses (Magnus, 1905), so probably *"in vivo"* the effect is due principally to an adrenal discharge. We have already mentioned that Cannon, Aub and Binger (1912) obtained inhibitory effects in cats with cava blood taken after nicotin injection only when the adrenals were present.

A donor (24 kgm.) received 2 mgm. nicotin. An adrenal discharge was

produced and registered by inhibition of the recipient's (10 kgm.) intestine. The blood pressure rose 198 mm. Hg. To obtain this rise it was necessary to inject 0.075 mgm. epinephrin. When small discharges were obtained,

TABLE 1

DATE, 1925	WEIGHT		DRUG INJECTED	DOSE	BLOOD PRESSURE VARIATION		INCREASE IN HEART RATE OF RECIPIENT
	Donor	Recipient			Donor	Recipient	
	kgm.	*kgm.*		*mgm.*	*mm. Hg*	*mm. Hg*	
4/4	19.0	9.0	Nicotin	19.0		+206	88
5/12	21.5	11.0	Nicotin	5.0	−40 +204	+174	112
5/15	25.2	11.0	Nicotin	5.0	+140	+200	106
5/16	23.5	16.0	Nicotin	2.5	+50	+190	66
5/26	15.0	10.0	Nicotin	1.0	+64	+170	76
5/27	17.5	9.0	Nicotin	0.01	0	0	0
5/27	17.5	9.0	Nicotin	0.1	+20	0	0
5/29	15.0	11.0	Nicotin	0.09	+30	0	0
5/30	19.0	8.5	Nicotin	1.0	−40 +200	+180	130
5/20	17.0	7.5	Cystosin	0.6	+40	+150	90
5/20	17.0	7.5	Cystosin	0.6	+48	+190	92
5/5	25.2	11.0	Lobelin	4.0	+170	+112	94
5/6	22.0	8.5	Lobelin	1.0	−70 +150	+140	84
5/18	18.0	8.0	Lobelin	2.0	−10 +130	+314	130
5/23	18.0	11.5	Lobelin	0.5	+130	+130	80
5/8	18.0	12.0	Coniin	5.0	+10	0	0
5/18	18.0	8.0	Coniin	10.0	−28	0	0
6/30	31.0	29.0	Coniin	20.0	+16	0	2
7/21	19.5	11.5	Tetramethylammonium chloride	34.0	−70 +44	+160	80
6/30	31.0	29.0	Tetramethylammonium chloride	33.0	0	+174	70
7/20	23.5	11.0	Tetramethylammonium bromide	10.0	0	0	0
7/21	19.5	7.0	Tetramethylammonium bromide	12.0	+56	+126	68
6/30	31.0	29.0	Tetramethylammonium iodid	40.0	0	+174	70
7/20	23.5	11.0	Tetrapropylammonium iodid	18.0	0	0	0
7/21	19.5	7.0	Hordenin sulphate	10.0	+22	0	0
7/21	19.5	7.0	Hordenin methyliodate	11.0	+186	+178	112
7/20	23.5	11.0	Piperidin	141	0	0	0
8/18	17.0	8.5	Tetrahydrobetanaphthylamin	68	+120	0	0

intestinal movements might be stimulated. Intestinal movements were registered by means of a small rubber bag filled with water, which was put into the upper part of the ileum, and communicated with a burette partially

filled with water. The changes in pressure were registered by a Marey tambour. The abdomen was left open so that contraction of the diaphragm and abdominal wall would not be a disturbing factor (fig. 3).

The inhibition observed cannot be attributed to nicotin, as the amount passing from the donor to the recipient is too small to produce any effect. Injection of a quantity of epinephrin equal to the amount discharged produces inhibition.

Cystosin inhibits intestinal contractions (Dale and Laidlaw, 1912). The amount of epinephrin discharged under the influence of this drug is capable of inhibiting the intestine. Nicotin does not have any inhibitory effect on the intestine of adrenalectomized animals. A dog (18 kgm.), whose intestine had been denervated by extirpation of the celiac plexus and section of the nerves accompanying the superior mesenteric artery, was given 1 mgm. nicotin. A rise in blood pressure was observed, accompanied by inhibition of peristalsis (fig. 4). Increased intestinal contractions were registered three and a half minutes later. After taking out the adrenals, 1 mgm., and a few minutes later, 2 mgm. nicotin were injected and no inhibition followed; on the contrary, an increase in tone and strong

TABLE 2

Glycemia per cent (Folin and Wu)

Donor	0.0694	0.0689	0.099	0.08	0.0694	0.0606
Recipient	0.0892	0.0869	0.103	0.121	0.1105	0.0819
Minutes after injection		15	30	45	60	75

contractions were observed. There were no variations in the blood pressure record.

Both these experiments show that the dose of nicotin injected produced effects on the intestine by means of an adrenal discharge. We have not tried larger doses which might inhibit the denervated intestine of adrenalectomized dogs.

Volume of the spleen. A marked contraction is registered in the spleen of the recipient during the adrenal discharge produced by nicotin injected in the donor (fig. 3).

Volume of the denervated limb. Only two experiments of this type were performed. In one Anrep's technique was followed. One milligram of nicotin given to a dog weighing 10 kgm. produced a marked rise of blood pressure and a short dilatation followed by a constriction of the denervated limb (Dale and Richards' plethysmograph).

A donor (18 kgm.) was given 1 mgm. nicotin, the blood pressure rising 70 mm. Hg. The recipient (9 kgm.) had a rise in blood pressure of 160 mm. Hg, the denervated heart increased in rate 70 beats per minute, the denervated limb, after a short dilatation, contracted markedly.

Effect on the blood sugar. On previous occasions we have shown that the simple anastomosis does not modify the blood sugar in either the donor or the recipient. But when nicotin is given to the donor, the blood sugar rises in both animals.

Protocol—Donor (19 kgm.). 1 mgm. nicotin injected intravenously. Recipient 9 kgm. Anesthesia: chloralose, 1 hour before injection of nicotin. Before transfusion the adrenal vein gave 11.4 cc. per minute. When transfusion ceased, it gave 1 cc. per minute. Blood pressure rose 210 mm. Hg in the recipient, the heart rate increased 88 beats per minute.

The effects observed are not due to nicotin passing from the donor to the recipient. Nicotin produces different effects in the donor and in the recipient. The donor has a less considerable increase in blood pressure and an initial slowing, followed by an irregular increase in the heart rate. The greater rise in blood pressure of the recipient is accompanied by an increased heart rate. But this is not sufficient evidence that both effects are not produced directly by nicotin, arriving in the recipient with the blood transfused.

To clear up all doubts in this respect, 1 to 2 mgm. nicotin were injected in the donor and the rise in blood pressure registered in the recipient. Transfusion was then interrupted and a second dose of nicotin given; 20 cc. of blood were taken from an artery a few seconds later and rapidly injected into the jugular vein of the dog which had recently been the recipient. No effects were seen on the blood pressure or the heart rate. When 2 mgm. were given, a slight effect was observed. Twenty cubic centimeters represent about twice the amount of blood that usually passes from one dog to another. If now the anastomosis is once more established, nicotin again produces a discharge in the donor registered by the recipient.

Protocols.

6/2/1925. Donor (24 kgm.). Recipient (8 kgm.). Anastomosis and 1 mgm. nicotin injected in the donor. Blood pressure in donor rose from 80 to 112 mm. Hg; in recipient from 90 to 180 mm. Hg. Heart rate increased from 90 to 108. Transfusion was interrupted and 6 minutes later 2 mgm. nicotin were injected in the donor; between 30 and 80 seconds after 20 cc. of arterial blood were collected and rapidly injected into the jugular of the recipient. Blood pressure of the donor rose from 80 to 170 mm. Hg and from 90 to 122 mm. Hg in the recipient; the heart rate increased from 88 to 100 in the latter. Transfusion was again established and 9 minutes later 2 mgm. nicotin given intravenously to the donor. The blood pressure rose from 80 to 100 mm. Hg in the donor and from 90 to 260 mm. Hg in the recipient, whose heart rate rose from 88 to 192 (fig. 5).

6/5/1925. Donor (23 kgm.). Recipient (9.3 kgm.). Transfusion interrupted and 1 mgm. nicotin injected intravenously in donor. Between 25 and 85 seconds after 20 cc. arterial blood collected and rapidly injected into the recipient's jugular. Blood pressure rose from 90 to 140 mm. Hg in the donor and remained 160 mm. Hg in the recipient. The heart rate was 116 before and 118 after the injection. Transfusion was again established and 1 mgm. nicotin given intravenously to the donor.

Blood pressure rose from 90 to 148 mm. Hg in the donor and from 160 to 280 mm. Hg in the recipient; the heart rate increased from 114 to 154.

These protocols show that when greater doses of nicotin are given to the donor, the amount passing to the recipient may be sufficiently large to produce slight effects concurring with the more marked effects occasioned by adrenal discharge. A simple calculation shows that the sharp increase in blood pressure is seen in the recipient when from 4 to 15 cc. only of the donor's blood has entered its circulation, that is to say, a dose of nicotin so small as to be ineffective.

Amount and duration of the discharge. The anastomosis method has two definite drawbacks for pharmacodynamic studies. The substance injected passes continuously in small amounts from one animal to the other and may produce direct effects on the recipient or reacting dog. Secondly, it

TABLE 3

DATE	WEIGHT	DRUG INJECTED INTRAVENOUSLY	MAXIMUM EPINEPHRIN SECRETION PER MINUTE AFTER INJECTION	EPINEPHRIN SECRETED PER KILOGRAM PER MINUTE
	kgm.	*mgm.*	*mgm.*	*mgm.*
6/6/1925	19		0.007	0.000368
		Nicotin 2	0.030	0.00157
		Cystosin 1	0.050	0.0026
		Lobelin 1	0.070	0.00368
6/8/1925	16		0.001	0.000062
		Nicotin 2	0.100	0.0062
		Lobelin 1	0.050	0.0031
6/9/1925	20		0.001	0.00005
		Nicotin 5	0.100	0 0050

gives no precise knowledge as to the quantitative variations occurring each minute in the amount of epinephrin secreted. Further experiments were necessary to fill this gap.

A dog was prepared in the same way as the donors of our previous experiments, but the anastomosis was not established. The adrenal blood was collected in graduated tubes containing 1 cc. of a 5 per cent sodium citrate solution and its adrenal content estimated on two dogs which had previously had their vagi cut and their stellate ganglia extirpated while under chloralose anesthesia and artificial respiration. The weight of the recipients was always from one-half to two-thirds that of the donor. Blood pressure and heart rate were recorded. Before injecting the adrenal blood their reaction to an 1:1,000,000 solution of epinephrin was tested and at the end of the experiment a second test was made with 1:100,000 solution and the blood-pressure raising effect of the different samples of blood com-

pared with that of the standard solution. Two dogs were used so as to give a sufficient interval between injections.

Nicotin (2 to 5 mgm.), cystosin (1 mgm.) and lobelin (1 mgm.) produced marked discharges for 1 to 2 minutes after the injections. After 2 or 3 minutes the normal level of secretion is restored; sometimes a period of diminished secretion follows the discharge. A second injection of the same or another epinephrin-secreting drug produces a second discharge. The amount secreted is considerable, 0.04 to 0.10 mgm. per minute for the left adrenal. This represents an increase of from four to one hundred times the normal quantity (table 3). The concentration also increased considerably—in one case, to 1:40,000.

TABLE 4

6/6/1925

	2 MGM. NICOTIN INTRAVENOUSLY					1 MGM. CYSTOSIN INTRAVENOUSLY				1 MGM. LOBELIN INTRAVENOUSLY			
Time after first injection, minutes.	–	1	2	3	4	5	16	17	18	25	26	27	28
Adrenal blood collected, cc	5	18	15	8	7	7	17	15	8.5	18	16	6	1
Epinephrin, mgm.	0.007	0.03	Trace			Trace	0.05	0.005	0.005	0.07	0.004		
Dog A, 14 kgm., rise in blood pressure, mm. Hg			0					18			7		
Increase in heart rate per minute			0					8			0		
Dog B, 15 kgm., rise in blood pressure, mm. Hg	22	98				0	142		4	140			
Increase in heart rate per minute	4	52				0	62		0	98			

Protocols.

6/6/1925. Dog (19 kgm.). Vagi cut in neck. Blood from left adrenal contained 0.004 to 0.0075 mgm. epinephrin per minute. After intravenous injection of 2 mgm. nicotin the epinephrin secreted in the first minute was 0.03 mgm.; from the second to the fifth minute the quantity of epinephrin in the samples was so small that it was impossible to estimate it with our method. Fifteen minutes after the nicotin injection 1 mgm. cystosin was given intravenously and 0.05 mgm. epinephrin was secreted during the first minute, 0.005 mgm. during the second minute, and 0.006 mgm. during the fifth minute. Nine minutes after the second injection, 1 mgm. lobelin was given intravenously, and 0.07 mgm. epinephrin was secreted during the first minute and 0.004 mgm. during the second minute (table 4, fig. 6).

6/8/1925. Dog (16 kgm.). Artificial respiration. Vagi not cut. Blood from left adrenal contained less than 0.001 mgm. epinephrin per minute. Intravenous injection of 2 mgm. nicotin gave 0.1 mgm. epinephrin during the first minute, and 0.045 mgm. during the fifth, eighth and tenth minutes. Lobelin (1 mgm.) was then

injected and again the secretion increased to 0.005 mgm. during the first minute, 0.05 during the second minute, and less than 0.001 during the sixth minute (table 5, fig. 7).

6/9/1925. Dog (20.5 kgm.). Vagi cut in neck. Artificial respiration. Epinephrin secreted by left adrenal was not more than 0.001 mgm. per minute. Nicotin (5 mgm.) intravenously injected increased the secretion to 0.07 mgm. during the first minute, 0.01 mgm. during the second minute; by the fourth minute the previous rate of secretion was again restored and at the seventeenth minute the amount secreted was a little less than before injecting the drug (table 6, fig. 8).

A great increase was observed in the adrenal circulation, which may be due to the general rise of blood pressure or to local vasomotor effects; this last cause has to be determined by other methods than the one we used. In some cases an increased adrenal secretion was recorded, although the circulation did not increase but actually diminished, therefore, it cannot be attributed to a better adrenal circulation.

Repeated injections. As has already been said, nicotin produces an adrenal discharge followed by a short period of diminished secretion. A second injection of 1 or 2 mgm. of the drug has the same effect when given 10 to 15 minutes after the first dose, and the observation can be repeated any number of times. If the interval between the injections is shortened, the discharge becomes gradually smaller, this being more marked the larger the doses and the shorter the intervals between them.

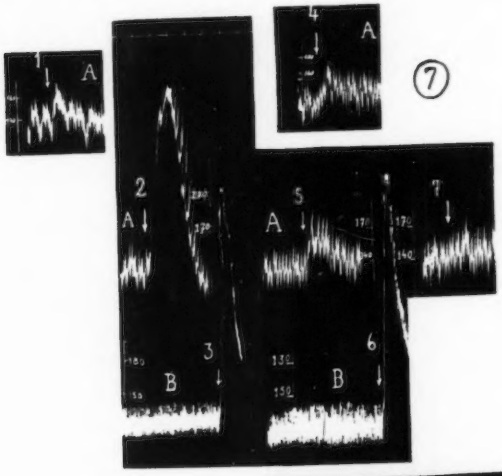
Protocol.

6/30/1925. Donor (19 kgm.). Recipient (8.5 kgm.). A preliminary stimulation of the left splanchnic produced an adrenal discharge. Fifteen nicotin injections were then given, as is seen in table 7 (fig. 9).

Fig. 7. 6/8/1925. Blood-pressure curves of test dogs A and B. *1.* Intravenous injection 8 cc. adrenal blood given by donor during 1 minute before injecting drugs. *2.* Intravenous injection 4 cc. adrenal blood given by donor during first minute after injecting 2 mgm. nicotin. *3.* Intravenous injection 10 cc. adrenal blood given by donor during second minute after injecting 2 mgm. nicotin. *4.* Intravenous injection 7 cc. adrenal blood given by donor during fifth minute after injecting 2 mgm. nicotin. *5.* Intravenous injection 2.5 cc. adrenal blood given by donor during the first minute after injecting 1 mgm. lobelin. *6.* Intravenous injection 8.2 cc. adrenal blood given by donor during second minute after injecting 1 mgm. lobelin. *7.* Intravenous injection of adrenal blood given by donor during sixth minute after injecting 1 mgm. lobelin.

Fig. 8. 6/9/1925. Blood-pressure curves of test dogs A and B. Small records. *1.* Injection of 0.003 mgm. epinephrin. *2.* Injection of 0.005 mgm. epinephrin. *3.* Injection of 11 cc. adrenal blood given by donor during 1 minute. Large record: *1.* Injection of 16 cc. adrenal blood given by donor during first minute after injection of 5 mgm. nicotin. *2.* Injection of 27 cc. adrenal blood given by donor during second minute after injection of nicotin. *3.* Injection of 7.4 cc. adrenal blood given by donor during fourth minute.

Fig. 9. 6/30/1925. Time in minutes. Blood-pressure curve of recipient (8.5 kgm.). Blood-pressure curve of donor (19 kgm.). At each arrow 1 mgm. nicotin injected intravenously into donor.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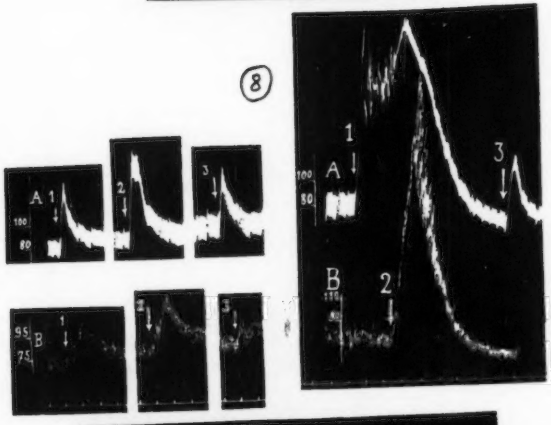
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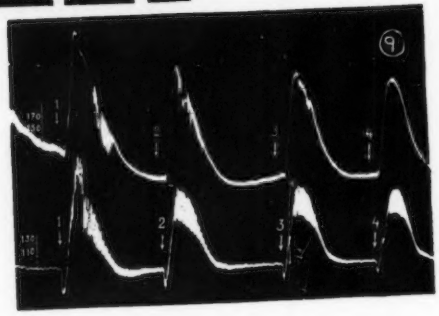
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TABLE 5

6/8/1925

	TIME, MINUTES																							
	—	2½	6	8	12	14	18	20½	30	32	45	53	54	55	56	57	58	63	64	67	102	108	116	122	130
Epinephrin, mgm.	0.003	0.003	0.005	0.005	0.008	0.008	0.012	0.012													0.05	0.10	0.02	0.075	0.1
Adrenal blood collected, cc.									8	8	7	5.5	4	10	9.8	7	4.2	25	9.2	5					
Epinephrin content, mgm.									<0.001	<0.001	0.001	—	0.1	0.045	—	0.002	0.002	0.005	0.05	<0.001					
Dog A, 9 kgm., rise in blood pressure, mm. Hg.	23		30		70		90		8				150			22		22		17	120		98	144	150
Increase in heart rate per minute	4		8		18		30		1				90			2		12		2	90		52	100	100
Dog B, 9 kgm., rise in blood pressure mm. Hg.		4		8		18		22		2	6			206			6		215			270			
Increase in heart rate per minute		6		12		16		26		2	6			110			0		104			124			

Blood pressure of A when experiment started 140 mm. Hg.
Blood pressure of B when experiment started 125 mm. Hg.
Pulse rate of A when experiment started 114 beats per minute.
Pulse rate of B when experiment started 98 beats per minute.
At 54 minutes 2 mgm. nicotin intravenously. At 63 minutes 1 mgm. lobelin intravenously injected.

TABLE 6

6/9/1925

	TIME IN MINUTES										
	0	2	6	9	15	17	1	2	3	4	14
							5 mgm. nicotin intravenously				
Epinephrin, mgm	0.003	0.003	0.005	0.005							
Adrenal blood collected, cc					11	11	16	27	13	7.4	7
Epinephrin content, mgm					0.001	0.001	0.07	0.1		0.001	0.001
Dog A, rise in blood pressure, mm. Hg	52			74	48		160			56	14
Increase in heart rate per minute	4			6	2		72				2
Dog B, rise in blood pressure, mm. Hg	30			40	20		250				
Increase in heart rate per minute	8			20	2		74				

Blood pressure of A at commencement 80 mm. Hg.
Blood pressure of B at commencement 75 mm. Hg.
Heart rate of A at commencement 76 beats per minute.
Heart rate of B at commencement 124 beats per minute.

TABLE 7

TIME	NICOTIN	BLOOD PRESSURE RISE	INCREASE IN HEART RATE BEATS PER MINUTE	EPINEPHRIN SECRETION
minutes	*mgm.*	*mm. Hg*		*mgm.*
0	1	180	134	0.01
9	1	175	94	0.01
19	1	160	74	0.09
27	1	145	94	0.07
34	1	150	14	0.01
39	1	154	84	0.08
44	1	120	40	0.04
49	1	120	50	0.04
56	1	120	60	0.04
61	2	145	78	0.06
66	2	65	26	0.01
71	2	78	36	0.02
76	2	120	58	0.04
84	5	110	60	0.03
89	5	20	8	0.01
Total				0.74

Four minutes after the last dose was given, strong stimulation of the left splanchnic of the donor produced only a slight discharge that gave a rise of blood pressure in the recipient of 20 mm. Hg. Nicotin (0.1 mgm. in 0.1 cc. water) was injected into the donor's left adrenal and a rise of blood pressure of 22 mm. Hg was registered in the recipient. The donor's left splanchnic was again stimulated and a marked rise of blood pressure (100 mm. Hg) was seen in the recipient. The donor's adrenal weighed 1 gm. and contained 0.33 mgm. epinephrin. The recipient had a rise of blood pressure of 165 mm. Hg and the heart rate was accelerated 84 beats per minute when 0.1 mgm. epinephrin was injected. A dose of 0.05 mgm. gave a rise of blood pressure of 135 mm. Hg and an increase in heart rate of 76 beats per minute. This experiment shows how difficult it is to suppress the effects of splanchnic stimulation and to exhaust the epinephrin content of the adrenals in the dog.

Continuous injection. By means of a Woodyatt pump, nicotin was injected continuously into a donor's jugular vein.

6/15/1925. Donor (24 kgm.). Recipient (10 kgm.). During 14 minutes 20 mgm. nicotin were injected in 200 cc. normal saline solution, that is to say, 1.428 mgm. per minute or 0.0595 mgm. per kilo per minute. Three minutes after the commencement of injection the donor's blood pressure rose suddenly, 80 mm. Hg in 1 minute.

Fig. 10. 6/15/1925. Time in minutes. Blood-pressure curve of recipient (10 kgm.). Blood-pressure curve of donor (24 kgm.). From *1* to *2* donor receives 1.428 mgm. nicotin per minute intravenously. From *2* to *5*, 4.28 mgm. nicotin per minute. *3*. Intense stimulation of left splanchnic during 10 seconds. *4*. Intense stimulation of left splanchnic during 20 seconds.

Fig. 11. 6/17/1925. Time in minutes. Blood pressure of donor (30 kgm.). Blood pressure of recipient (11 kgm.). From the arrow onwards donor receives during 10 minutes 12 mgm. nicotin in 150 cc. normal saline.

Fig. 12. 6/19/1925. Time in minutes. Blood pressure of recipient (9.5 kgm.). Blood pressure of donor (30 kgm.). From *1* to *4* donor received in 20 minutes 10 mgm. nicotin in 250 cc. normal saline; from *4* to *5*, 10 mgm. nicotin in 10 minutes; from *5* onwards 9.5 mgm. nicotin in 15 minutes.

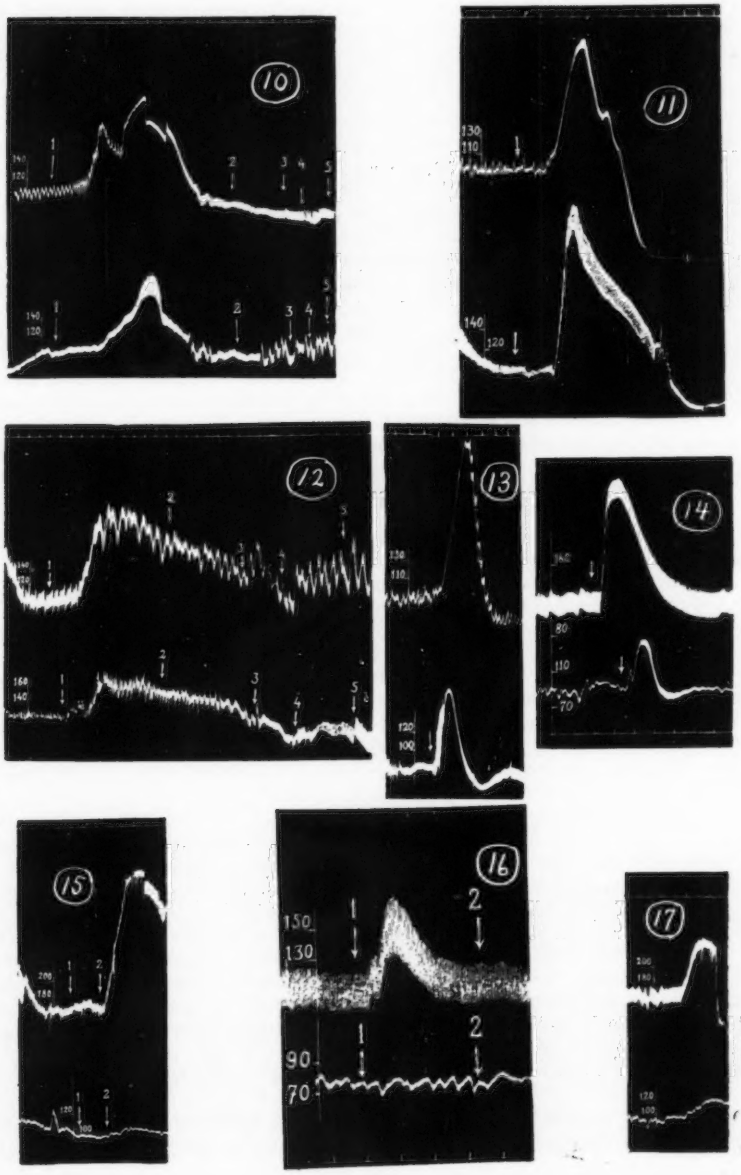
Fig. 13. 6/2/1925. Time in minutes. Blood-pressure curve of recipient (5 kgm.). Blood-pressure curve of donor (24 kgm.) with left adrenal denervated. At the arrow 1 mgm. lobelin intravenously injected into donor.

Fig. 14. 7/21/1925. Blood-pressure curve of recipient (11.5 kgm.). Blood-pressure curve of donor (19.5 kgm.) with left adrenal denervated. Time in minutes. At the arrow intravenous injection to donor of 12 mgm. tetramethylammonium bromide hydrate.

Fig. 15. 6/5/1925. Time in minutes. Blood-pressure curve of recipient (9.3 kgm.). Blood-pressure curve of donor (23 kgm.). *1*. Injection of 0.1 cc. nomal saline into donor's adrenal. *2*. Injection of 0.15 mgm. nicotin in 0.1 cc. normal saline into donor's adrenal.

Fig. 16. 7/20/1925. Blood-pressure curve of recipient (11.0 kgm.). Blood-pressure curve of donor (23.5 kgm.). Time in minutes. *1*. Injection of 0.0001 mgm. nicotin into donor's adrenal. *2*. Injection of 0.00001 mgm. nicotin into donor's adrenal.

Fig. 17. 6/5/1925. Time in minutes. Blood-pressure curve of recipient (9.3 kgm.). Blood-pressure curve of donor (23 kgm.). Donor's semilunar ganglion painted with a 1 per cent solution of nicotin.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then, after a slight fall, a second rise of 110 mm. Hg was recorded; after that the blood pressure fell and a third short rise was followed by a rapid fall. The blood pressure remained above the normal level for 9 minutes, a duration never obtained with single injections. The recipient's blood pressure rose gradually 3 minutes after the commencement of injection and reached a first peak in the fourth minute; after a slight fall in the sixth minute, a second peak was attained in the eighth minute (110 mm. Hg): the blood pressure then gradually fell, returning to the original level in the sixteenth minute. During the fourteenth minute a second solution of greater strength was injected, so that 30 mgm. nicotin in 200 cc. saline were given in 7 minutes, that is to say, 4.28 mgm. per minute or 0.178 mgm. per kilo per minute. No effect was registered in donor or recipient. Four minutes after the second injection was started, the donor's left splanchnic was strongly stimulated for 10 seconds and 2 minutes later for 30 seconds. No effect was observed in either of the dogs (fig. 10).

6/17/1925. Donor (30 kgm.). Recipient (11 kgm.). In 10 minutes, 12 mgm. nicotin in 150 cc. normal saline (1.20 mgm. per min. or 0.040 mgm. per kgm. per min.) were injected in the donor's jugular vein. Two minutes and 45 seconds after starting the injection a sharp rise of blood pressure was observed in the donor; the peak (170 mm. Hg) was reached in 3 minutes; then the blood pressure fell rapidly reaching 0 in the eighth minute and the animal died during the tenth minute after injection. The recipient's blood pressure rose rapidly 45 seconds after the donor's had started to rise and reached a peak of 180 mm. Hg. Then it fell slowly, returning to its original level in the tenth minute. A maximum increase in heart rate of 78 beats per minute was registered during the first six minutes of the rise of blood pressure (fig. 11).

6/19/1925. Donor (30 kgm.). Recipient (9.5 kgm.). In 20 minutes 10 mgm. nicotin in 250 cc. normal saline (0.5 mgm. per min. or 0.0167 mgm. per kgm. per min.) were injected into the donor's jugular. The donor's blood pressure started to rise 45 seconds after commencement of injection and reached a peak of 70 mm. Hg in three and one-half minutes. Then it fell slowly and reached the previous level in the eighteenth minute after injection. The recipient's blood pressure started to rise the third minute and reached a peak of 120 mm. Hg between the fourth and fifth minutes. A gradual fall brought the pressure to the previous level twenty minutes after injection. The rapidity of the injection was now doubled so that 10 mgm. nicotin in 250 cc. normal saline were given to the donor in 10 minutes. The donor's blood pressure rose only 10 mm. Hg; the recipient's was maintained between 40 and 80 mm. Hg above the base line. A third injection of 9.5 mgm. nicotin in 115 cc. normal saline given in 15 minutes produced no results in either dog; neither did a fourth injection of 40 mgm. nicotin in 40 cc. normal saline given in 4 minutes alter the blood pressure of donor or recipient. The prolonged rise when nicotin was injected slowly is remarkable, since we had never before observed it to last so long.

Before the first injection was started, the donor's left splanchnic was stimulated and a rise of blood pressure of 60 mm. Hg was registered in the recipient. During the injections the splanchnic was again stimulated 19, 23, 28, 31, 34 and 38 minutes after the first injection. A rise of blood pressure always resulted in both a donor and the recipient. The last stimulation gave a rise of 30 to 40 mm. Hg in the recipient. Here again it was impossible to paralyze the splanchnic (fig. 12).

The following conclusions can be drawn from the three experiments just described: *A*, nicotin produces no effect until a certain quantity has entered the circulation; *B*, once this minimal quantity has arrived, the

rise of blood pressure is sharp in both animals; *C*, although a continuous stream of nicotin enters the circulation, it is effective for only 8 or 10 minutes in either dog; *D*, the vasomotor and adrenal secreting effects of splanchnic stimulation can only be suppressed with great difficulty.

Adrenal extirpation. Adrenalectomized dogs show the same rise of blood pressure when nicotin is injected as normal animals. A dog weighing 23 kgm., and which had had both adrenals extirpated, gave a rise of blood pressure of 206 mm. Hg after the injection of 10 mgm. nicotin. A second adrenalectomized animal (20 kgm.) showed a rise of blood pressure of 190 mm. after injection of 5 mgm. nicotin. This does not mean that epinephrin plays no part in the rise in blood pressure, but simply that it is not the sole factor in its production. In an intact animal a vasomotor and an adrenal mechanism work together; when the adrenals are suppressed, only the vasomotor factor is present and in transfusion experiments only the adrenal mechanism is active in the recipient.

It is remarkable that the rise of blood pressure is generally greater in the recipient that receives the discharge of one adrenal only, than in the donor that has both the other adrenal and the vasomotor mechanism active. A possible explanation of this is that nicotin diminishes the response of the muscles of the blood vessels.

The importance of the adrenal factor is seen in the following experiment. The celiac plexus of an animal is taken out. The injection of 1 mgm. nicotin produces a rise of blood pressure of 180 to 200 mm. Hg. Then the adrenals are extirpated and injections of 1, 2 and 5 mgm. nicotin give no rise in blood pressure. Either the nervous mechanism (celiac plexus) or the humoral mechanism (adrenals) must be present to obtain a rise in blood pressure after nicotin injection. If only one of them is suppressed, the other acts vicariously; when both are destroyed, no effect is observed.

INFLUENCE OF NERVOUS SYSTEM. It is a common observation that destruction of the central nervous system does not suppress the rise in blood pressure produced by nicotin.

Section of the splanchnics and adrenal denervation. The section of the major splanchnic does not suppress the discharge in the homolateral adrenal (experiment of 6/15/1925). Bilateral section of the major and minor splanchnics, extirpation of the left abdominal sympathetic chain and the right adrenal does not suppress the discharge of the left adrenal after nicotin (experiments of 4/17/1925 and 4/18/1925). The same is true when the right adrenal is extirpated and the left denervated (experiment of 4/20/1925). The extirpation of the left adrenal and the semilunar ganglion does not suppress the discharge in the right adrenal when cystosin is given (experiment of 5/4/1925). Complete denervation of the left adrenal does not suppress its discharge after injections of nicotin, cystosin, lobelin (fig. 13), hordenin methylicdate and tetramethylammnoium iodid. In

these experiments to be absolutely sure that no nervous connections with the adrenals remained, the celiac ganglion was removed and all the nerve structures around the hepatic, superior mesenteric and renal arteries were destroyed. The adrenal was dissected so that it was only attached by the lumbo-adrenal vein and a few small arteries. This diminished the adrenal circulation somewhat—0.5 to 1 cc. blood per minute flowing from the lumbo-adrenal vein. Epinephrin injections in the donor sometimes caused a slight rise of blood pressure in the recipient, especially when the adrenal

TABLE 8

Adrenals denervated

DATE, 1925	WEIGHT		DRUG INJECTED		RISE OF BLOOD PRESSURE		INCREASE IN HEART RATE RECIPIENT BEATS PER MINUTE	TOTAL EPINEPHRIN DISCHARGED
	Donor	Recipient			Donor	Recipient		
	kgm.	*kgm.*		*mgm.*	*mm. Hg*	*mm. Hg*		*mgm*
4/15	17.5	9.0	Nicotin	17.5	124	200	130	0.2
4/17	18.0	7.0	Nicotin	18.0	90	40	42	0.1
4/18	19.0	10.0	Nicotin	11.0	90	190	122	0.1
4/20	19.0	5.3	Nicotin	10.0	210	170	88	0.06
6/1	15.5	12.5	Nicotin	2.0	94	160	34	
6/2	24.0	5.0	Nicotin	2.0	108	166	84	0.04
6/2	24.0	5.0	Nicotin	1.0	26	90	18	0.03
5/4	16.5	8.0	Cystosin	16.5	60	140	122	0.075
6/1	15.5	12.5	Cystosin	1.0	68	130	44	
6/1	15.5	12.5	Lobelin	0.5	60	124	38	
6/2	24.0	5.0	Lobelin	1.0	88	172	92	0.04
7/24	17.5	15.5	Hordenin sulphate	10.0	20	0	0	
7/24	17.5	15.5	Hordenin sulphate	20.0	24	0	0	
7/24	17.5	15.5	Hordenin methyliodid	10.0	140	132	60	
7/24	17.5	15.5	Tetramethylammonium iodid	10.0	50	50	18	
7/24	17.5	15.5	Hordenin sulphate	10.0	20	0	0	
7/24	17.5	15.5	Hordenin sulphate	20.0	24	0	0	
7/24	17.5	15.5	Hordenin methyliodid	10.0	140	132	60	
7/24	17.5	15.5	Tetramethylammonium iodid	10.0	50	50	18	
7/24	17.5	15.5	Tetrabetahydronaphthylamin	20.0	0	0	0	

circulation was very slow (0.25 cc. per minute), but never produced the considerable increases seen after nicotin, cystosin or lobelin. Asphyxia, stimulation of the medulla, etc., produced no rise of blood pressure in the recipient. It can, therefore, definitely be stated that nicotin and the other drugs studied produce their effects through a real increase in the epinephrin secretion and not by a simple alteration of the circulation.

Ergotoxin. As this drug paralyzes the endings of the sympathetic and suppresses or inverts the effects of its stimulation or those of epinephrin

injection we thought it would be of interest to study the effects on the nervous mechanism of adrenal secretion.

Protocol.

5/16/1925. Donor (23.5 kgm.). Recipient (16 kgm.). Epinephrin (0.05 mgm.) was injected into the donor, whose blood pressure rose 170 mm. Hg. No effect was observed on the recipient. Three injections of 25 mgm. ergotoxin were made with 5 and 3 minute intervals between them. The same dose of epinephrin now gave a rise of only 70 mm. After 40 mgm. more of ergotoxin, epinephrin still gave a rise of 30 mm. Hg. With 2 to 3 minute intervals 4 injections of 25 mgm. ergotoxin were given and epinephrin still produced a rise of 30 mm. The donor's left splanchnic was stimulated and its blood pressure rose only 20 mm. whereas the recipient's showed a considerable increase (190 mm.); proof that an adrenal discharge had been produced. Five subsequent doses of 25 mgm. ergotoxin suppressed all rise of blood pressure in the donor after splanchnic stimulation, but did not interfere with the adrenal discharge, as a rise of 60 mm. was recorded in the recipient. Nicotin (5.2 mgm.) was then injected into the donor, whose blood pressure rose 40 mm.; the recipient's blood pressure went up 190 mm. owing to an adrenal discharge.

TABLE 9

DATE, 1925	WEIGHT		NICOTIN INJECTED INTO THE ADRENAL	BLOOD-PRESSURE VARIATION		INCREASE IN HEART RATE OF RECIPIENT
	Donor	Recipient		Donor	Recipient	
	kgm.	*kgm.*	*mgm.*	*mm. Hg*	*mm. Hg*	
6/ 5	23.0	9.3	0.15	0	180	66
6/ 5	23.0	9.3	0.15	0	90	58
7/20	23.5	11.0	0.0001	0	50	40
7/20	23.5	11.0	0.00001	0	0	0

This experiment shows us that ergotoxin does not interfere with the action of nicotin on adrenal secretion. It also seems to indicate that the nerve endings of the splanchnics in the adrenals are not paralyzed by ergotoxin when the vaso-constrictor fibers have already been blocked.

Injection into the adrenal gland. The experiments described seemed to show that nicotin, cystosin and lobelin have a directly stimulating action on the adrenal medulla.

Injection of saline solution (0.1 to 0.15 cc.) directly into the adrenal medulla of a donor produced no effects in the recipient (six experiments). When 0.1 or 0.15 cc. of a 0.1 per cent nicotin solution is injected in exactly the same conditions, a marked adrenal discharge is immediately observed (two experiments).

Protocol.

6/5/1925. (Fig. 15) Donor (23 kgm.). Recipient (9.3 kgm.). An injection of 0.15 cc. of an 0.9 per cent sodium chloride solution was made into the donor's left adrenal. No change in blood pressure or pulse rate was registered in either dog. Three minutes later 0.15 cc. of a 0.1 per cent solution of nicotin in normal saline

was injected in the same way. The donor's blood pressure rose 6 mm. Hg; the recipient's pulse rate increased 64 beats per minute and its blood pressure rose 120 mm. After an interval of 42 minutes the injection of normal saline again produced no change. A second injection of nicotin, given as before, did not alter the donor's blood pressure or pulse rate, but raised the recipient's blood pressure 80 mm. Hg and increased the pulse 58 beats per minute.

The minimal effective dose of nicotin that produces an adrenal discharge when injected into the adrenal medulla is very small. We obtained a discharge with 0.0001 mgm. and not with 0.00001 mgm. (see fig. 16). Many other drugs have been studied but no one was nearly as potent as nicotin in this respect. (These experiments will be referred to in a later paper.)

Action on the semilunar ganglion. Nicotin produces its effects in a complex way. It acts on the central nervous system, on the sympathetic ganglia and directly on some tissues. It has been maintained that the nerve fibers that stimulate adrenal secretion arrive at the medulla without a relay in the ganglia. Elliott (1913) is of this opinion, having observed that section of the splanchnics is followed by degeneration of medullated fibers in the adrenal medulla, where nerve cells are very rarely found.

The semilunar ganglion was carefully isolated from the surrounding tissue by means of rubber membranes and then painted with a 1 per cent solution of nicotin. The donor's blood pressure rose only 3 mm., but the recipient's rose 76 mm. and the heart rate increased 66 beats per minute. Both responses lasted for 4 minutes (fig. 17).

Nicotin, therefore, stimulates the cells of the semilunar ganglion and apparently a relay does exist here in the adrenal secretory path. Only two sources of error are possible in our experiment; first, the nicotin might have been absorbed and thus have entered the adrenal by way of the blood or lymph; second, the diffusion of the drug would lead to the direct stimulation of the adrenal. Precautions were taken to avoid diffusion, so this second source of error can be practically disregarded.

We have confirmed Langley's observation on the difficulty of paralyzing the dog's sympathetic by means of nicotin.

Localization of the action of nicotin on adrenal secretion. Probably nicotin starts the secreting impulse in the central nervous system, the semilunar ganglion and the adrenal medulla.

The stimulation of the adrenal centers may be admitted, since nicotin has a diffuse stimulating effect on the central nervous system. In the present paper no proof has been given that this is so. It would be necessary to perfuse the head with nicotin solutions or to inject them into the carotid arteries. Under any other experimental conditions, peripheral effects come into play.

The stimulation of the celiac ganglion apparently occurs as the experi-

ments of painting it with nicotin show. This fact has some importance as a proof that at least some of the splanchnic fibers have a relay station in the ganglion before passing to the adrenal. Elliott's (1913) contention that all the fibers go directly to the medulla cells without a peripheral synapse cannot be maintained. At the most some fibers may follow an uninterrupted course, the others are relayed. Stewart and Rogoff had admitted *a priori* the direct action of nicotin on the celiac ganglion, but they offered no proof of this assertion.

The direct action of nicotin on the adrenal medulla, a tissue closely related embryologically with the sympathetic ganglia, had been suspected by various authors. Eichholtz (1923) showed that an increased adrenal secretion was present after nicotin injections in animals whose splanchnics had been severed. Our experiments prove that not only nicotin, but cystosin and lobelin produce an adrenal discharge when the gland is completely denervated. Eichholtz did not extirpate the celiac ganglion so that the results of his experiments may be attributed to a direct effect on the ganglion and not on the adrenal. A complementary proof is given by the experiments in which an adrenal discharge is obtained by injecting the drug directly into the gland.

SUMMARY

1. Using Tournade and Chabrol's method of suprareno-jugular anastomosis, it has been observed that nicotin, cystosin, lobelin, hordenin methyliodid and quaternary ammonias produce a marked adrenal discharge, generally lasting a short time.

2. The discharge is not due to the increase of blood pressure observed in the donor.

3. The effects observed are not due to the passage of the drug through the anastomosis from the donor to the recipient.

4. The adrenal discharge produces the following effects in the recipient: marked increase in blood pressure, great acceleration of the heart rate, inhibition of the intestine, contraction of the spleen or the denervated limb, and hyperglycemia.

5. The peak in the discharge occurs between the first and third minute, and can be as much as 0.10 mgm. per minute for one gland. Three to five minutes later the normal level or a lower one is established.

6. Repeated injections produce repeated discharges but when the injections are made at short intervals and the dose is large, the effects diminish in intensity. Continuous injections produce a continuous discharge, but of decreasing amounts.

7. The discharge is produced after the central nervous system has been destroyed, the splanchnics cut or the gland has been thoroughly denervated.

8. A discharge is obtained when the celiac ganglion is painted with nicotin.

9. Nicotin, cystosin, lobelin and quaternary ammonias stimulate the adrenal medulla directly.

10. Nicotin produces its characteristic effects, rise of blood pressure and retarding of heart rate, in adrenalectomized dogs.

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SPECTROPHOTOMETRIC DETERMINATIONS OF BILIRUBIN

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In the work previously reported by us it was shown that, by means of the spectrophotometer, bilirubin may be detected in the colorless plasma of the normal dog. Furthermore, spectrophotometric determinations have furnished the basis for the accumulation of data to show that bilirubin is made from hemoglobin in the spleen and bone marrow.

General descriptions and discussions relative to our operative, chemical and spectrophotometric procedures are to be found in earlier communications. There are, however, besides these investigations, various data and conclusions with reference to the spectrophotometric determinations of bilirubin which we believe may be of value to others who are interested in such experiments. In the course of our work we have found that in time there is a fading of solutions of bile and of blood serum and plasma which is evidenced by the increased amount of transmission of light in the general absorption region, 430 to 500 mμ. Without doubt the effects of fading enter into the spectrophotometric determinations of a series of solutions of bile or of blood plasma and serum, since it may require several hours to obtain the complete curves. From observations on the relationship between the increase of transmission of light and the time in hours subsequent to the removal of the material to be examined, we have been able to obtain certain experimental criteria which we believe should be observed if experimental data are not to be called into question. Furthermore, after these relationships have been determined, it is possible to obtain curves which show that certain physical laws are strictly applicable to the experimental data obtained.

EFFECTS OF FADING, OR INCREASE OF SPECTROPHOTOMETRIC TRANSMISSION WITH TIME. Figure 1, A and B, contains two sets of spectrophotometric curves for the absorption region 430 to 500 mμ and shows the relations between the transmission of light for the various wavelengths specified and the time in hours after the removal of the bile from the gall bladder. The data with regard to the periods of time elapsing after the bile was secured, and the time at which the various spectrophotometric

readings were made are noted in the legends attached to figure 1. Both groups of curves were obtained with bile from the gall bladder of normal dogs. Initially the dilution was made with alcohol to the point corresponding to the limit obtainable with the Van den Bergh method. The degree of dilution was further modified until both samples, when put in containing cells of 10 cm. capacity, gave the same spectrophotometric reading (20 per cent transmission) at wavelength 440 mμ. In figure 2 are plotted curves for these two solutions of bile, designated as A and B, and for the wavelengths 440 and 470 mμ respectively, showing the relationship between the percentage transmission of light, or degree of fading, and the time in hours subsequent to the withdrawal of the original sample of bile. These curves show that there is a rapid rise in the transmission of light during the first six to eight hours and that thereafter the rate of increase of transmission diminishes and becomes nearly linear with time. It is also obvious that the degree of fading is greater in the case of sample A than it is in sample B. These data and other confirmatory evidence lead us to conclude that 1, there is a rapid rate of fading, as evidenced by the increase in the transmission of light, in solutions of bile or blood plasma and serum during the first few hours subsequent to their removal from the body; 2, a decided reduction in the rate of fading occurs at approximately six to ten hours after removal and thereafter the degree of fading, or the percentage of transmission of light, becomes approximately linear with time; 3, it is highly desirable that various samples of blood which are to be compared for bilirubin content should be centrifuged simultaneously and otherwise cared for and treated in an identical manner, and that spectrophotometric determinations should not be attempted until about eight to ten hours have elapsed; and 4, spectrophotometrically it is impossible to compare accurately samples of bile or serum taken from different sources unless the readings are made soon after removal from the body and as rapidly as possible. Experience has shown that these various sources of inaccuracy in spectrophotometric methods for the determination of bilirubin may be avoided in large measure by the following simple procedure.

Fig. 1. The curves, designated as *A* and *B*, show the relationships between the transmission of light for the wavelengths specified and the time in hours after the removal of the bile from the gall bladders of normal dogs, and the time at which the various spectrophotometric readings were made.

A. Curve 1, 3 hours; curve 2, 6 hours; curve 3, 11½ hours; curve 4, 24 hours; curve 5, 60 hours; curve 6, 80 hours.

B. Curve 1, 2¾ hours; curve 2, 6¼ hours; curve 3, 12 hours; curve 4, 24½ hours; curve 5, 60½ hours; curve 6, 79 hours.

Fig. 2. Curves showing the relationships between the percentage transmission of light and the time after removal of the bile from the gall bladder for solutions *A* and *B*, used in getting the data of figure 1, and for wavelengths 440 mμ and 470 mμ. These curves illustrate the effects of fading, which occurs with time.

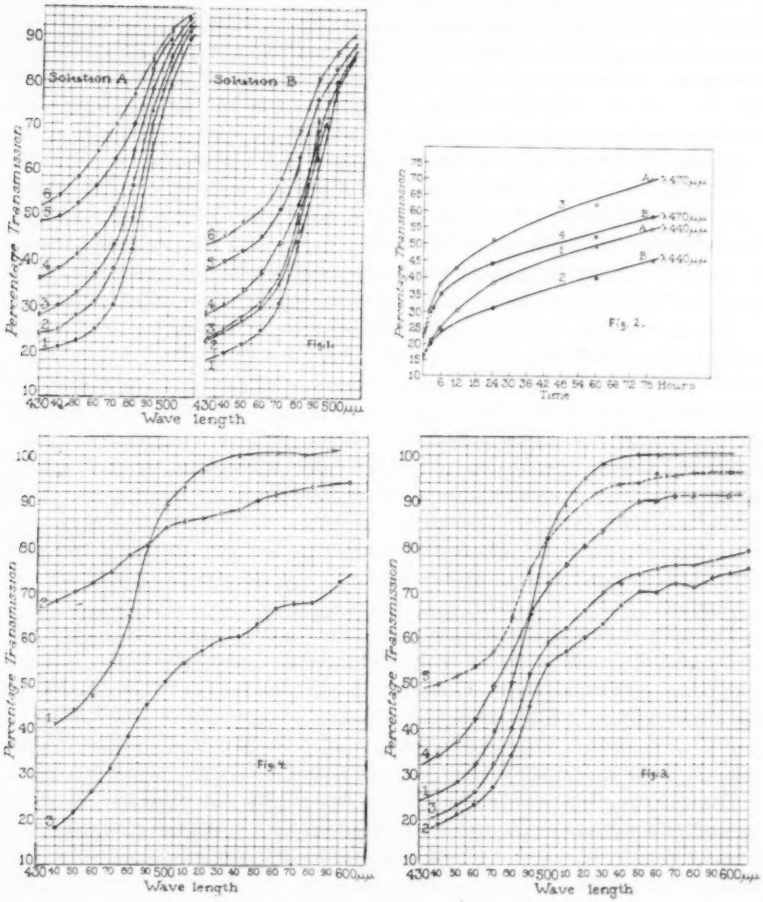

Fig. 3. Curves showing the effects of small amounts of hemolysis on the spectrophotometric data. Curve 1, portion of the original bile solution *A* after standing 29 hours; curve 2, spectrophotometric data after the introduction of 0.5 cc. of a solution of 1 cc. of dog blood in 1000 cc. of alcohol into the contents of the 10 cm. tube used in obtaining curve 1; curve 3, after standing 4 hours; curve 4, 18 hours; curve 5, 102 hours.

Fig. 4. Illustrating the effects of slight degrees of hemolysis on the spectrophotometric determinations of bilirubin. Curve 1, original bile solution *B* after standing 50 hours; curve 2, spectrophotometric curve from a 10 cm. length of solution consisting of 1 cc. of blood to 30000 cc. of alcohol; curve 3, showing the effects of the addition of 2 cc. of the contents of the foregoing (curve 2) to the solution shown in curve 1.

Various samples of blood, taken from the same animal and in which it is desired to compare the amounts of bilirubin present, are collected and centrifuged, and the necessary quantities of solution prepared by sufficient but equal dilutions in all cases. These solutions are kept for from six to ten hours under identical conditions. The containing tubes are then filled simultaneously. Each solution is introduced in turn into the spectrophotometer and the readings of wavelength and percentage transmission in the region of 430 to 500 mμ are made as rapidly as possible. It is necessary, however, to obtain additional data on the percentages of light transmission for wavelengths from 500 to 680 mμ, for the reasons that 1, if the transmission in the regions of longer wavelengths is not at least 90 per cent there is evidence that the solution is not photometrically sufficiently clear, which, as we know from our experimental tests, indicates that the determinations in the absorption zone 430 to 500 mμ will be affected and therefore be of doubtful value for comparative purposes; and 2, we must look for evidences of hemolysis, which will be indicated by the presence of slightly increased absorption or reduced percentages of light transmission in the regions of 580 mμ and 540 mμ respectively (the absorption zones of hemoglobin). If the spectrophotometric data show the presence of any hemolysis, the readings obtained in the region 430 to 500 mμ cannot be used unequivocally, for the reason that the presence of any hemolysis lowers the percentages of transmission in the blue-violet region of the spectrum.

The instrument which we have used in our work as previously reported and which was employed in these experiments is known as the Keuffel and Esser color analyzer and has been particularly serviceable because of the speed with which determinations can be made. There are, however, retinal phenomena, such as adaptation, sensitiveness and fatigue, which enter into the ease, speed and accuracy with which data can be obtained. We have found that undesirable factors making for inaccuracies and slowness of reading can be eliminated to some extent by using the instrument in a fairly darkened room, with only sufficient auxiliary light to read the photometer and wavelength scales, and with the observing eye exposed and adapted to radiation from the absorptive region under examination. To illustrate: we have generally proceeded to set on 430 mμ and to read the photometer, discarding the first few readings or waiting until the point is reached at which two or three successive readings are in very close agreement. Without doubt, these phenomena vary for different observers, and as a result small differences in readings at the same wavelength will be consistently obtained by different observers. This emphasizes the importance of one and the same observer making all determinations on any set or group of solutions from which the data are to be compared. Through numerous checkings we have found that any differences which

may exist in the spectrophotometric determinations of the amounts of bilirubin in a series of specimens of blood serum, for example, as determined by one experienced observer, can also be obtained by a second competent observer, although the one curve will rarely, if ever, be exactly superimposed on the other.

EFFECTS OF SLIGHT DEGREES OF TURBIDITY AND HEMOLYSIS. We have previously called attention to certain necessary experimental criteria in spectrophotometric measurements. These criteria are the clearness of the solution and its freedom from hemolysis. In order to show the significance and importance of the standards of clarity and freedom from hemolysis which have been adopted, there is shown in figure 3 a set of curves which is fairly representative of the effects we have encountered in our experiments. Curve 1 represents the spectrophotometric readings obtained with the original sample of bile from a normal gall bladder, initially diluted with alcohol to about the limit of accuracy of the Van den Bergh method and read after it had stood twenty-nine hours. After curve 1 had been obtained, 0.5 cm. of the solution in the tube of 10 cm. length used in obtaining curve 1 was replaced by 0.5 cc. of a solution of 1 cc. of dog blood in 1000 cc. of alcohol. The content of blood in the tube in terms of the original concentration was therefore about 1:22,000. Curve 2 shows the presence of a slight amount of hemolysis, as indicated by the dips in the curve at 580 and 560 mμ respectively, and also shows the reduction in absorption in the region 430 to 500 mμ. Therefore it would appear to be illogical to make comparisons between two curves such as curves 1 and 2 in a series of experiments having to do with variations in the amount of bilirubin. And again, the transmissions in the regions of longer wavelength, from 540 to 600 mμ, for example, are decidedly reduced in value as compared with the transmissions in the same region given by the initially tested solution. After the solution had stood for four hours, curve 3 was obtained. In this curve the presence of hemolysis is not shown by increased absorption in the regions of 580 and 540 mμ, but the turbidity is clearly indicated by the relatively low readings (between 70 and 80 per cent) in the region of 540 to 600 mμ. Curves 3, 4 and 5 show that the fading effects, discussed herein, also occur, and that there is, with time, a marked increase in the percentage of transmission of light in the region 430 to 500 mμ. Curve 4 also shows the fact that the percentage transmission in the region 430 to 500 mμ is greater than for the original solution (curve 1), while the power of transmission of the combined solutions is less in the region 540 to 600 mμ when compared with the readings for this same spectral region, as given by the original solution.

In figure 4, curve 1 shows the original solution of bile from a normal gall bladder, diluted to the limit of accuracy of the Van den Bergh test, after standing fifty hours; curve 2 shows a solution of 1 cc. of dog's blood

in 30,000 cc. of alcohol, and curve 3 shows the addition of 2 cc. of the foregoing solution to the solution of bile shown by curve 1 after the withdrawal of an equal quantity. The alcoholic solution of blood was slightly turbid as is shown by curve 2. The addition of 2 cc. of the solution used for curve 2 to that of curve 1 shows in curve 3 a marked decrease in transmission throughout the whole of the spectrum. The amount of decrease in the power of transmission, however, is greater in the region 500 to 600 mμ than in the region 430 to 500 mμ.

It seems probable from these data that the presence of turbidity may be, in part at least, an indicator of slight hemolysis. This hemolysis may be too slight to evidence itself by the presence of increased absorption at 580 and 540 mμ.

These results, therefore, show that the criteria of clarity of solution and freedom from hemolysis are most important if correct conclusions are to be drawn from experiments of the character which we have already reported, and on which further work is being done. We feel that we have not set too high a standard when we say that, as a general rule, comparisons of amounts of bilirubin in blood serum or plasma should not be attempted in any set of experiments unless the spectrophotometric readings in the long wavelength end of the visible spectrum (540 to 700 mμ) are approximately 90 per cent, and that the readings for all curves in any set used for drawing conclusions should be comparable to each other in this region. It should be pointed out, however, that when turbidity or hemolysis is present there is always a greater reduction in the power of transmission in the regions of long visual wavelength than there is in the absorption region 430 to 500 mμ. As a result, therefore, it is probable that one may be justified in drawing conclusions as to the increase or decrease of amount of bilirubin present from a series of curves in which the changes in transmission of light in the zone 430 to 500 mμ are considerably greater than they are in the region from 540 to 700 mμ. However, the optimal conditions are those which we have outlined and should be adhered to as closely as possible.

Comparison of sensitiveness of the spectrophotometric method with the Van den Bergh method. In order to get an idea of the sensitiveness of the spectrophotometric determinations as compared with the Van den Bergh method, samples of bile were taken from the gall bladders of normal dogs and diluted to approximately 0.000001 gram of bilirubin for each cubic centimeter, that is 0.1 mgm. bilirubin for each 100 cc. as determined by the Van den Bergh method. The numerical quantities which we have specified are said to be approximately the limit of accuracy of that method. Figure 5 shows a series of spectrophotometric curves of bile from the gall bladder of a normal dog after successive dilutions with alcohol. The curve marked C = 100 is taken as having 100 per cent

concentration, and is the spectrophotometric curve representative of the probable limit of the Van den Bergh method. An inspection of the curves of figure 5 shows that it is possible to determine the character and shape of the spectrophotometric curve of bilirubin for a dilution as low as one-fiftieth (C = 2) of the smallest amount measurable by the Van den Bergh method. From a comparison of the spectrophotometric determinations in the region 430 to 480 mμ for known dilutions of bile we have shown in our previous papers that we were dealing with quantities of bile pigment in the blood plasma or serum of dogs varying from 0.04 to 0.015 mgm. for each 100 cc. of solution.

Application of the laws of Lambert and Beer. In order to be able to compare the amount of light absorbed by one substance or solution with that absorbed by another, it is necessary to establish some standard of measurement. For this purpose Bunsen and Roscoe introduced the extinction coefficient, symbolized by ϵ. From Lambert's law, $\epsilon = -\frac{\log I}{X}$ in which I represents the intensity of the transmitted light and X the depth of solution. From Beer's law, $\frac{C_1}{C_2} = \frac{\epsilon_1}{\epsilon_2}$ hence the absorption of light by different concentrations of the same substance in the same solvent is directly proportional to the concentration, C. In all of our investigations we have used tubes of 10 cm. length, hence X, the depth of solution, is a constant. Therefore, if the laws of Lambert and Beer are applicable to the work in which we are engaged,

$$\frac{C_1}{C_2} = \frac{\epsilon_1}{\epsilon_2} = \frac{-\log I_1}{-\log I_2} = \frac{\log I_1}{\log I_2}$$

In figure 6 we have plotted on semilogarithmic paper the concentrations as abscissae, and percentages of light transmission as ordinates, for the various dilutions of bile from the gall bladder of a normal dog as given in figure 5 and for wavelengths 430, 450, 470 and 480 mμ, respectively. The relationship between C and $\log I$ is, in each instance, a linear one as should be the case if the laws of Lambert and Beer are applicable.

Furthermore, these lines pass quite exactly through the point marked 100, indicating that, with zero concentration of pigment in solution, there is no absorption of light and that the absorption zone has completely disappeared. In general, however, it is unlikely that one will obtain curves in which the logarithm of the transmitted light varies accurately in proportion to the concentration for the reason that fading, which occurs with time of standing and which has been considered in other portions of this paper, causes an increase in the percentage of light transmission. In order to get sets of results similar to those given in figure 6, in which all lines pass through the 100 per cent point for zero concentration of bili-

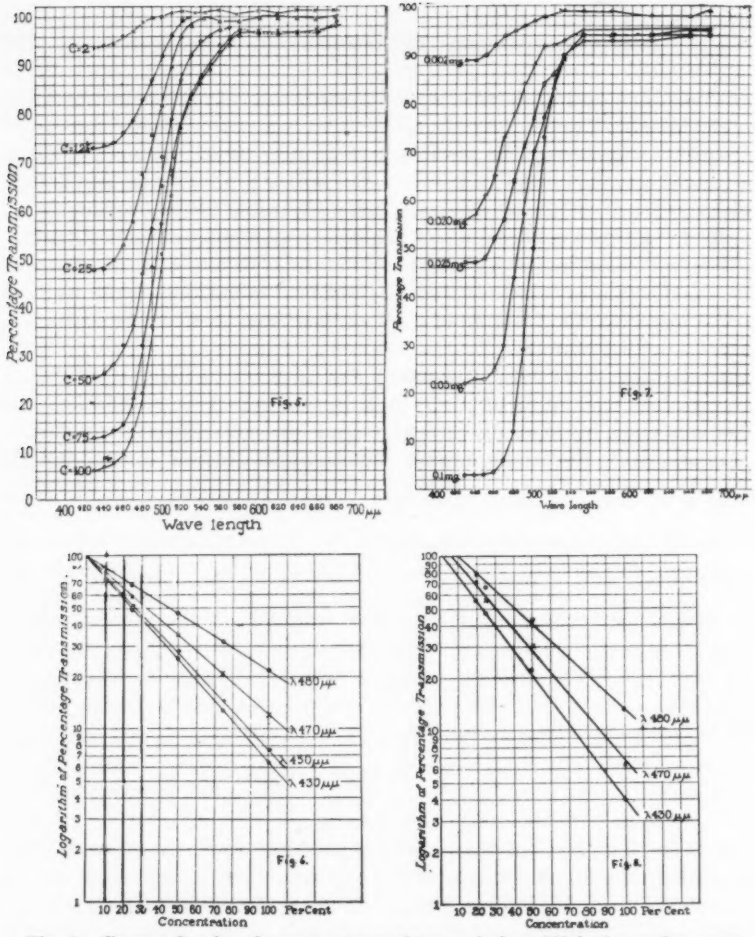

Fig. 5. Curves showing the percentages of transmission of light for various wavelengths with changes in the concentration of bile originally taken from the gall-bladder of a normal dog. The curve marked $C = 100$ is representative of spectrophotometric data obtained with absorption cells 10 cm. in length at the limit of the Van den Bergh method.

Fig. 6. Curves showing that, under the special procedures outlined in this paper, the laws of Lambert and Beer can be applied with accuracy to the absorption of light by solutions of varying concentration for wavelengths in the absorption zone of 430 mμ to 500 mμ.

Fig. 7. Curves showing the percentages of transmission of light for various wavelengths as the dilution of the solution of bile is changed. The curve marked 0.1 mgm. gives the data obtained with a solution of bile representative of the limit of accuracy of the Van den Bergh method.

Fig. 8. Curves showing the results of the application of the laws of Lambert and Beer to the data of figure 7. The curves for the three wavelengths chosen show that fading has occurred, as is evidenced by the fact that none of the lines pass through the 100 per cent point.

rubin, it is necessary to take a series of containing tubes of equal length, fill them with their respective solutions as made up from the original which has stood for several hours prior to dilution, and then make the readings of wavelength-transmission as rapidly as possible. Unless these precautions are observed one will get a series of curves similar to those shown in figure 8, which are in turn plotted from data shown in figure 7. In this instance, the data were obtained, in part, within two to three hours after the withdrawal of the bile from the gall bladder and the procedures were contrary to those which we have stated to be optimal. The curves of figure 8 show that the relationship between the concentration and the logarithm of the transmitted light is approximately a linear one, but that, because of fading, the lines fail to pass through the 100 per cent point.

SUMMARY

We have presented and discussed the effects of fading and slight degrees of turbidity and hemolysis on the spectrophotometric determinations of bilirubin in blood plasma or serum. The laws of Lambert and Beer can can be applied with accuracy to the absorption (or transmission) of light in bile from the gall bladder of a normal dog in the region 430 to 500 mμ provided the experimental procedure is such as to minimize the effects due to fading. It is possible to determine quite accurately by the spectrophotometric methods the presence of bilirubin in serum to one-fiftieth (possibly as a limit, one-hundredth), of the smallest amount measurable by the Van den Bergh method.

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THE EFFECT OF EXTERNAL TEMPERATURE ON SECOND WIND

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It has been demonstrated that artificial hyperthermia is accompanied by a hyperpnea (Haldane, 1905; Hill and Flack, 1907) while the considerable rise of rectal temperature that occurs during muscular work is also well established (Cook and Pembrey, 1913). It therefore seemed possible that the hyperpnea at the commencement of muscular work might be influenced by body temperature changes, a possibility already considered by Campbell, Douglas and Hobson (1919), and the following experiments were undertaken to obtain evidence for or against such an hypothesis. In particular attention was given to the actual rate of change of body temperature in exercise, since in most researches such temperature measurements have been made only at infrequent intervals. Benedict and Slack (1911) did take continuous records of rectal temperature during exercise in three experiments (finding it to remain constant in two cases and to rise in the other), yet they did not employ heavy work, nor did they record the respiratory changes. Campbell, Douglas and Hobson recorded the respiratory changes seen after work of 1056 kilogram meters per minute in two experiments of about twenty minutes duration and noted a rise of rectal temperature of rather less than 0.5°C. in each case, but they did not record the changes in temperature during the actual work and they used two fans to assist the subject to cool while working on the bicycle. In observations on second wind, MacKeith, Pembrey, Spurrell, Warner and Westlake (1923) noticed a rise of rectal temperature of 1.0°F. accompanying second wind, but again no attempt was made to determine the rate of change.

METHODS USED. The work was performed on a bicycle ergometer of the Tissot type, the rate being regulated by a metronome and determined by a cyclometer. The rectal and axillary temperatures were both recorded by resistance thermometers and a Gamgee thread recorder of the type described by Woodhead and Varrier Jones (1916). The actual thermometer used for the axilla (6×0.7 cm. in size) was packed into the cavity, surrounded by cotton, and the arm was then secured to the body with a rubber bandage; a closed cavity was thus formed. Without doubt

the axillary temperatures thus recorded were accurate. The rectal temperature was determined similarly, using the ordinary Woodhead-Jones model (11.0 × 0.9 cm.) inserted to a depth of 8 cm. so that the sphincter gripped the groove. While the thermometer would be reasonably accurate if used for a subject in bed, the protruding silver tube, though covered while in use with rubber tubing and cotton, is apt to be affected by the room conditions, and probably records a temperature slightly below the real rectal temperature. In order to check and control these results a few experiments (three) were performed with thermocouples (steel-constantan) in the axilla and rectum (latter to 8.0 cm. depth), determining the temperature from the galvanometer deflection when these were opposed to a couple maintained at a known temperature in a thermos flask. The sensitivity was such that 1.0 mm. on the scale corresponded to about 0.05°C. Both resistance thermometers and thermocouples were

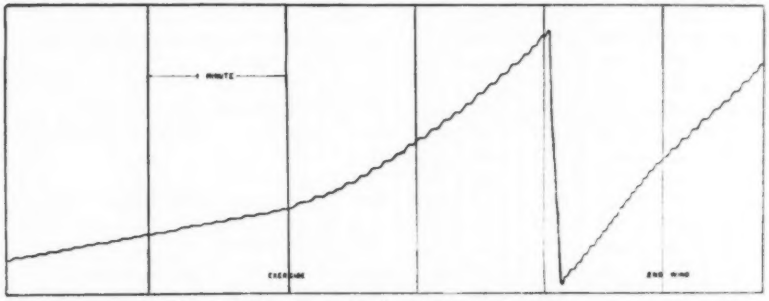

Fig. 1. Tracing made from a typical record.

carefully standardized against a thermometer immersed in water within a thermos flask every day.

The respiratory ventilation was measured by making the subject inspire from a large spirometer of the Tissot type (Harvard pattern) through rubber flutter valves. The spirometer movements were recorded by the method employed by Landis, Long, Dunn and Meyer (1926). As soon as the spirometer was emptied it was filled rapidly by a connection with a compressed air supply, so that only a few moments were lost. Parts of a typical record are reproduced in figure 1. Through a signal magnet the time in which the subject had sensations of second wind was also recorded.

The subject sat on the bicycle for fifteen minutes to allow the axillary temperature to reach an equilibrium, and a record of the volume of air breathed while resting (four minutes) was taken; exercise was then commenced and continued usually until four minutes after the subjective sensation of second wind; respiratory and temperature records were con-

tinued for a further four minutes or longer. The temperature conditions of the room were recorded, including kata thermometer readings. As a rule the subject wore only light underclothing (B.V.D.'s), but in two experiments heavier clothing was worn. In all, 19 experiments on five subjects were made.

RESULTS OBTAINED. *Second wind.* The subjects noticed, as a rule, a very definite sensation of second wind, the respirations becoming easier, and the actual performance of the muscular work requiring less effort.

TABLE 1

NUMBER	SUBJECT	RATE	WORK	SECOND WIND	DRY BULB TEMPERATURE	WET BULB TEMPERATURE	KATA		REMARKS
							Dry	Wet	
			kg.-m.	*minutes*					
1	G.	172	311	2.62	25.0	14.3	3.80	15.0	Fan during work
2	G.	171	310	None	14.3	8.4	19.3	35.0	
3	G.	150	260	4.50	21.8	13.1	6.48	14.8	
4	G.	140	253	3.25	27.5	15.5	3.44	11.95	
5	G.	150	237	2.92	24.5	16.1	3.90	12.7	Heavy clothing
6	G.	150	237	3.06	24.8	18.0	3.82	12.40	
7	N.	218	444	1.57	25.6	16.8	3.37	12.2	
8	N.	151	410	3.42	25.0	14.3	3.80	15.0	Fan during work
9	N.	162	278	3.97	27.5	15.5	3.44	11.95	
10	N.	150	272	3.08	23.3	15.4	4.25	12.60	
11	N.	151	205	3.25	22.7	15.2	4.48	12.7	Heavy clothing
12	J.	175	870	5.40	25.0	14.3	3.80	15.0	Fan during work
13	J.	176	837	3.50	25.6	16.8	3.37	12.2	
14	J.	159	727	3.88	21.5	10.5	4.77	16.35	
15	J.	154	628	6.67	25.2	15.5	3.72	12.8	
16	J.	155	613	None	24.8	16.6	3.75	11.9	Respiratory change at 5.92
17	B.	174	475	2.88	18.8	11.4	4.74	15.2	
18	B.	151	238	3.67	24.8	18.0	3.82	12.46	
19	D.	162	256	None	24.8	18.0	3.82	12.46	Respiratory change at 12.22

The relative importance of the above two subjective factors seemed to vary in different subjects and even in different experiments on the same subject. It was at once noticeable that this sensation appeared earlier and was more definite, the greater the rate of work, and it also became evident that it occurred earlier in a warm room than in a cold one, and probably earlier also when the subject was more heavily clothed. Indeed in a very cold room with light underclothing no second wind might be recognizable.

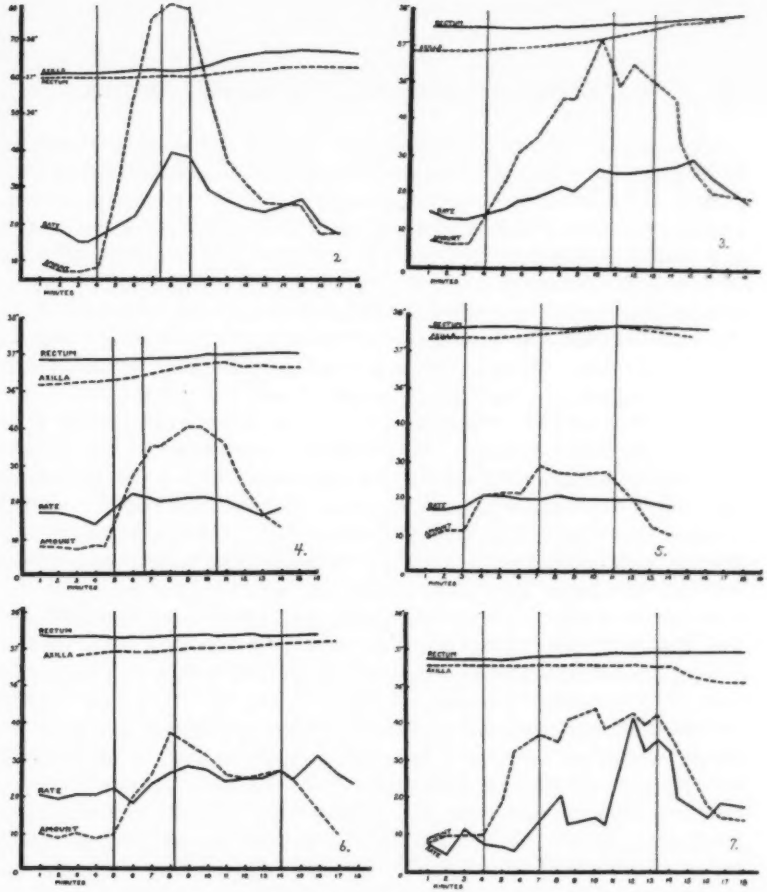

Fig. 2. Usual conditions. Rate 176, work 837 kg.-m., second wind in 3.50 minutes, dry bulb 25.6, wet bulb 16.8, dry kata 3.37, wet kata 12.2. Clothing B.V.D. Subject J. Resistance thermometers. (The three vertical lines represent the beginning of exercise, second wind, and end of exercise, respectively; *axilla* is axillary, and *rectum*, rectal temperature in degrees C.; *rate* represents number of breaths per minute, and *amount* volume of air breathed in liters per minute measured at prevailing room temperature, etc., in this and all subsequent figures.

Fig. 3. Usual conditions. Rate 154, work 628 kg.-m., second wind in 6.67 minutes, dry bulb 25.2, wet bulb 15.5, dry kata 3.72, wet kata 12.8. Clothing B.V.D. Subject J. Thermocouples.

Fig. 4. Usual conditions. Rate 218, work 444 kg.-m., second wind in 1.57 minutes, dry bulb 25.6, wet bulb 16.8, dry kata 3.37, wet kata 12.2. Clothing B.V.D. Subject N. Resistance thermometers.

Fig. 5. Usual conditions. Rate 162, work 278 kg.-m., second wind in 3.97 minutes, dry bulb 27.5, wet bulb 15.5, dry kata 3.44, wet kata 11.95. Clothing B.V.D. Subject N. Thermocouples.

Fig. 6. Usual conditions. Rate 140, work 253 kg.-m., second wind in 3.25 minutes, dry bulb 27.5, wet bulb 15.5, dry kata 3.44, wet kata 11.95. Clothing B.V.D. Subject G. Thermocouples.

Fig. 7. Heavy clothing. Rate 150, work 237 kg.-m., second wind in 2.92 minutes, dry bulb 24.5, wet bulb 16.1, dry kata 3.90, wet kata 12.7. Subject G. Resistance thermometers.

Table 1 gives the results obtained in general. The effect of varying work under fairly similar temperature conditions is demonstrated by comparison of experiments 7 with 9, and of 13 with 15. That changed temperature conditions also affect the time is demonstrated by comparison of 1 with 2, 7 with 8, and 12 with 13. It will be noted that in the last two pairs of experiments the time of second wind is postponed by the use of a fan blowing on the subject during the time the actual work was done; the kata thermometer readings were taken under the resting conditions without the fan. Comparison of 4 with 5, and 9 with 11 shows how heavier clothing may quicken the onset of second wind.

Respiratory changes. These were in general of the same type as those already recorded by Krogh and Lindhard (1913) and by Barr (1923). The respiration rose evenly to reach a maximum which as a rule occurred just before the subject signalled second wind. After this the volume dropped to a more or less constant value, though often there was a secondary rise sometimes to a value above that occurring before second wind, and yet this later hyperpnea might not be associated with the distress felt previously. There was often a marked correspondence between the respiratory change and the time of subjective second wind, demonstrating an interdependence or common origin, but such a correspondence was not invariable; instances were seen where the respiratory records showed the usual changes and no second wind was noted, as well as the above mentioned instances where the respiration rose to still greater volumes after second wind was experienced. The view advanced by Pembrey and his associates that second wind is *entirely* a matter of relief of respiratory distress was not thus sustained. The actual changes occurring are illustrated in figures 2, 3, 4, 5, 6 and 7. The effect of the amount of work on the time of development of second wind is well shown in figures 2 and 3, and in figures 4 and 5, each pair representing a single subject. Figure 4 also illustrates how the respiratory volume may continue to rise even after subjective sensations of second wind have occurred. Figures 6 and 7 represent still another subject and demonstrate the effect of heavy clothing; though second wind occurred somewhat earlier, other factors are introduced,—the respiratory volume and respiratory rate remained at an unusually high value, and the subject was distressed.

Changes in axillary and rectal temperature. Figures 3, 5 and 6 give the changes observed in the experiments when thermocouples were used. When the work is high and the room relatively warm there is a rise both of rectal and axillary temperature, but second wind usually occurred at a time when there was no change in rectal temperature or a rise of only 0.05°C. On the other hand, the axillary temperature in nearly every case was rising rapidly at this time. The values shown in the other records were obtained with the resistance thermometers and show much the same

changes except that in figure 2 the rectal temperature appears to lie at a level below that of the axilla. This is probably due to the error of the rectal thermometer, to which reference has already been made. In some experiments with the resistance thermometers the rectal temperature actually appeared to fall during work, but such a fall was not observed when thermocouples were used; presumably the fall was due to some change exaggerating during work the cooling of the rectum by the loss of heat along the silver tube of the thermometer. When work was done in a warm room wearing heavy clothing (see fig. 7) the rectal temperature might rise, even with comparatively light work.

It was noticeable that the subjects might commence to sweat when no rise of rectal temperature exceeding 0.05°C. was demonstrable, even with thermocouples. In the experiment plotted in figure 6 the subject did not begin to show any noticeable sweating until 8 minutes from the commencement of work. On the other hand, in the experiment shown in figure 5 sweating occurred one minute before second wind was signalled. The close correspondance between sweating and second wind observed by Pembrey and Cooke (1913) was not seen in our experiments.

Discussion of results. The subjective sensation of second wind unquestionably comes earlier the warmer the room conditions and the heavier the work done. The subjective sensations are confirmed by records of the respiratory changes, which show similar effects. The increase in the volume of air breathed with exercise occurs more rapidly in a warm than in a cold room. Neither of these variations appears to be dependent on changes in deep body temperature in the trunk, since at this time the rectal temperature is scarcely altered.

Since it has been demonstrated by Zondek (1919) that the temperature of the muscles in the limbs is considerably below that of the rectum, the most reasonable hypothesis seems to be that the rate at which the muscle develops its full power to remove lactic acid is determined by its temperature, and that the temperature of the muscle reaches its optimum earlier when the metabolism is high with heavy work, or when the surrounding air is warm.

CONCLUSIONS

Second wind develops earlier, the warmer the room and the heavier the work done. It may be developed at a time when the rectal temperature has shown no change, and it is suggested that variations in the muscle temperature may be a factor. Sweating may occur before the rectal temperature shows any appreciable rise.

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STUDIES ON EPILEPSY

III. The Influence of a General Increase or Diminution of Intracranial Pressure upon the Susceptibility of Animals to Convulsive Seizures[1]

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In many epileptic seizures a rise in general blood pressure occurs before the onset of the convulsive movements, while in others a fall in blood pressure has been observed. In some patients with intracranial tumor in whom a decompressive operation has been performed, an increase of tension within the skull with more bulging and tenseness of the cerebral hernia occurs before the beginning of a convulsive seizure. These and other considerations have led the writers to investigate experimentally the possible relationship between a rise or fall of intracranial pressure and the susceptibility to convulsive seizures.

The injection into the blood stream of animals of large quantities of hypotonic solutions is known to cause a marked rise in general intracranial pressure and during operations upon the brain, the same increase of tension has been observed to follow a large intravenous infusion of normal salt solution.

On the other hand, as has been shown by the researches of Weed and McKibben, Foley, Howe and others, and by clinical experiences at the bedside and the operating table, a general fall of intracranial pressure can be brought about by the intravenous injection of hypertonic solutions. In animal experiments the use of hypotonic and hypertonic solutions is therefore a useful method by which intracranial pressure can be raised or lowered.

The investigations herewith reported were made upon adult cats in which convulsions can be produced by the intravenous injection of essence

[1] This study was made possible through a grant for a research on epilepsy from the Commonwealth Fund of New York to the New York Neurological Institute, for which we desire to express our gratitude.

of absinth. The amount of the convulsing agent that will cause a convulsive seizure and the character of the convulsions have been described in previous papers.[2]

It was shown that the intravenous injection of 0.035 to 0.04 cc. of the solution per pound of animal would, in many animals, cause tonic and clonic convulsions, and this was considered a "normal convulsing dose" for cats.

I. THE SUSCEPTIBILITY OF CATS TO THE CONVULSIVE AGENT AFTER A GENERAL RISE OF INTRACRANIAL PRESSURE HAS BEEN PRODUCED BY THE INTRAVENOUS INJECTION OF DISTILLED WATER OR BY THE INTRACRANIAL INJECTION OF VASELINE OR PARAFFIN. In a number of experiments 100 to 150 cc. of distilled water were injected intravenously before the animals received the absinth solution. In all of these experiments, the convulsing dose was much smaller than that for normal animals. A few minutes after the injection of the hypotonic solution there was a general hyperactivity of the tendon reflexes and the convulsions which followed the injections of absinth solution were often severe and distinctly prolonged.

Instead of the normal convulsing dose of 0.035 to 0.04 cc. per pound of animal, convulsions often followed the injection of 0.02 to 0.025 cc. per pound of animal. Two protocols follow:

Experiment 377. Adult cat, weight 8½ pounds

2:40 p.m. Intravenous injection of 100 cc. of distilled water.

	Amount of absinth injected	*Result*
2:56 p.m.	0.17 cc. or 0.02 cc. per pound	Severe and prolonged convulsions
3:06 p.m.	0.17 cc. or 0.02 cc. per pound	Marked convulsive twitches
3:11 p.m.	0.21 cc. or 0.025 cc. per pound	Two severe convulsions
3:17 p.m.	0.21 cc. or 0.025 cc. per pound	Severe convulsion with opisthotonus and extensor spasm

Experiment 378. Adult cat, weight 4½ pounds

3:45 p.m. Intravenous injection of 150 cc. of distilled water

	Amount of absinth injected	*Result*
4:13 p.m.	0.13 cc. or 0.025 cc. per pound	Pupils dilated, respiratory dyspnea, convulsive twitches
4:18 p.m.	0.13 cc. or 0.025 cc. per pound	A number of severe convulsions during six minutes
4:26 p.m.	0.14 cc. or 0.03 cc. per pound	Two severe convulsions
4:30 p.m.	Animal killed	

[2] Elsberg and Stookey: Arch. Neurol. and Psychol., 1923, ix, 613. Pike and Elsberg: This Journal, 1925, lxxii, 337.

The same results were observed when the general intracranial pressure was raised by injecting paraffin or vaseline into the cranial cavity.

Experiment 388. Adult cat, weight 9¾ pounds

3 cc. of paraffin injected extradurally through small opening in skull over left frontal region.

	Amount of absinth injected	*Result*
2:34 p.m.	0.195 cc. or 0.02 cc. per pound	Marked respiratory effect
2:44 p.m.	0.195 cc. or 0.02 cc. per pound	Two severe convulsions
3:06 p.m.	0.25 cc. or 0.025 cc. per pound	
3:17 p.m.	0.25 cc. or 0.025 cc. per pound	Convulsive twitches
3:25 p.m.	0.25 cc. or 0.025 cc. per pound	Convulsive twitches
3:35 p.m.	0.25 cc. or 0.025 cc. per pound	Severe convulsion
3:39 p.m.		Death

The post-mortem examination showed that the paraffin lay extradurally over a large part of the left cerebral hemisphere. There was some extradural hemorrhage under the paraffin.

II. THE SUSCEPTIBILITY OF CATS TO THE CONVULSING AGENT, AFTER INTRACRANIAL PRESSURE HAS BEEN REDUCED BY THE INTRAVENOUS INJECTION OF HYPERTONIC SOLUTIONS. In order to diminish intracranial pressure a series of animals received intravenously 100 cc. of 5 per cent sodium chloride solution. To determine that the results obtained were not due directly to the salt used, another series of animals received intravenous injections of 100 cc. of 25 per cent glucose solution. The results from sodium chloride and glucose were identical: the dose of absinth necessary to produce a convulsion was raised so that it often required doses of 0.05 to 0.06 cc. per pound of animal to produce a convulsion.

III. THE AVERAGE TOTAL AMOUNT OF ABSINTH REQUIRED TO CAUSE A CONVULSION, AND THE AVERAGE TOTAL AMOUNT THAT WILL CAUSE A FATAL CONVULSION, IN ANIMALS WITH INCREASED OR DIMINISHED INTRACRANIAL PRESSURE. As was to be expected, there is a certain degree of variation in the susceptibility of animals to the convulsing agent. We have, therefore, examined the results we obtained in series I and II from the viewpoint of the average total dosage of absinth required to produce the results. To arrive at the figures in the following tables, the combined weight of all the animals used for each series of experiments was divided by the number of the animals, and the average weight of animal recorded. Likewise the total amount of absinth used for each series of experiments was divided by the number of the animals and the average amount used for each animal of average weight was thus obtained. This amount was again divided by the average weight of the animals and the average dose of absinth per pound of animal thus determined.

a. The total amount of absinth required to cause the first convulsion.

TOTAL WEIGHT IN POUNDS	TOTAL AMOUNT OF ABSINTH TO FIRST CONVULSION	AVERAGE WEIGHT OF ANIMALS IN POUNDS	AVERAGE AMOUNT OF ABSINTH TO FIRST CONVULSION	AVERAGE DOSES OF ABSINTH PER POUND OF ANIMAL
1. Ten normal cats				
62.25	4.64 cc.	6.23	0.464 cc.	0.075 cc.
2. Six cats. Intracranial pressure raised by intravenous injection of 100 cc. of distilled water or hypotonic solution				
37.0	1.75 cc.	6.1	0.291 cc.	0.047 cc.
3. Seven cats. Intracranial pressure lowered by intravenous injection of 100 cc. of hypertonic solution				
40.25	5.48 cc.	5.75	0.782 cc.	0.136 cc.

These figures show that compared with normal animals the average total amount of absinth necessary to produce the first convulsion was much smaller in the animals in which intracranial pressure had been raised by the intravenous injection of hypotonic solutions, while, on the contrary, the average total amount of absinth necessary to produce the first convulsion was much larger in those animals in which a fall of intracranial pressure had been produced by the intravenous injection of hypertonic solutions. In the cats with increased intracranial pressure the average total convulsing dose per pound of animal was about one-half of that for normal animals, while in the cats with lowered pressure the average convulsing dose was almost twice that necessary in normal animals.

b. The total amount of absinth required to cause a fatal convulsion.

TOTAL WEIGHT IN POUNDS	TOTAL AMOUNT OF ABSINTH TO FIRST CONVULSION	AVERAGE WEIGHT OF ANIMALS IN POUNDS	AVERAGE AMOUNT OF ABSINTH TO FIRST CONVULSION	AVERAGE DOSES OF ABSINTH PER POUND OF ANIMAL
1. Four cats. Intracranial pressure raised				
29.5	5.56 cc.	7.37	1.39 cc.	0.188 cc.
2. Six cats. Intracranial pressure lowered				
34.0	14.02 cc.	5.66	2.33 cc.	0.412 cc.

These figures show that in the animals with increased intracranial pressure, an average of 0.188 cc. of absinth per pound of animal was required to cause a fatal convulsion, while on the other hand, in the animals with lowered intracranial pressure an average of 0.412 cc. per pound of animal—or almost three times as much—was required for the same result.

SUMMARY AND CONCLUSIONS

The susceptibility of cats to convulsive seizures from absinth was increased whenever intracranial pressure was raised by the intravenous injection of distilled water or the intracranial injection of vaseline or paraffin, and much smaller doses of the convulsing agent were required to produce tonic and clonic convulsive seizures. On the other hand, the susceptibility of the animals was diminished after intracranial pressure had been lowered by the intravenous injection of hypertonic solutions.

There are, without doubt, other factors which have an influence upon the susceptibility of the brain to initiate those motor discharges that we call convulsions. The above described experiments show that variations in intracranial pressure have a decided effect, although it is as yet impossible to state whether this is due to the increase or decrease of intracranial pressure itself or occurs secondarily through an effect upon the circulation of blood (and perhaps of other fluids) in the motor areas of the cortex and the subcortical pathways.

It may well be that the benefit from starvation in the treatment of some cases of epilepsy is due to some extent at least to the decrease in intracranial pressure consequent upon the diminution in the fluid intake.

Unilateral convulsive seizures—the so-called Jacksonian epilepsy—may be due to a considerable degree to localized variations in intracranial pressure. We will, in the near future, report upon the results of experiments in which an increase of pressure over strictly localized areas of the brain was produced, and will, in that connection, give evidence in support of a new explanation for generalized and for Jacksonian convulsive seizures in man.

A STUDY OF THE VAGO-ENTERIC MECHANISM BY MEANS OF NICOTIN

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While studying the effect of massive doses of nicotin on the enteric nervous mechanism, we had occasion to stimulate the vagi after giving very large doses of nicotin, e.g., 0.5 to 1.0 gram of the alkaloid per kilo of body weight. The responses of the intestine to vagus stimulation were not abolished by these large doses of nicotin, but, on the contrary, were greater and more constant than before nicotin was administered. This finding is apparently contrary to the generally accepted conclusion of Bayliss and Starling (1899) that "All effects of the vagus on the intestine are completely and, in our experiments, permanently abolished by minimal doses of nicotin, e.g., 0.3 cc. of a 1 per cent solution." Therefore, further investigation of the reaction of the intestine to vagus stimulation following the administration of nicotin seemed desirable.

METHODS. The present series consists of twelve experiments on etherized dogs. No morphin or other drugs were used in connection with the anesthesia. Both splanchnic and both vagus nerves were severed; the latter in the thorax below the origin of their cardiac branches. The peripheral ends of both vagus nerves were placed on electrodes of the Sherrington type. The thoracic incision was closed around the wires and the pneumothorax reduced. In two of the experiments the vagi were cut and stimulated in the neck in order to avoid any possible spread of the current to the esophagus. The abdomen was opened in the mid-line and a balloon placed in a convenient loop of the small intestine through an incision in the intestinal wall aboral to this loop. Records were obtained by means of a piston recorder with air transmission.

The nicotin used in all but three experiments was prepared as a 1 per cent or 5 per cent solution of the alkaloid and neutralized to a pH of about 7.6 with hydrochloric acid. To avoid error due to a possible difference between the effects of the hydrochloride and the alkaloid a 10 per cent solution of the alkaloid was used without neutralization in three experiments. The vagi were stimulated by means of an interrupted induced current obtained from a Porter inductorium with two storage cells in series in the primary circuit.

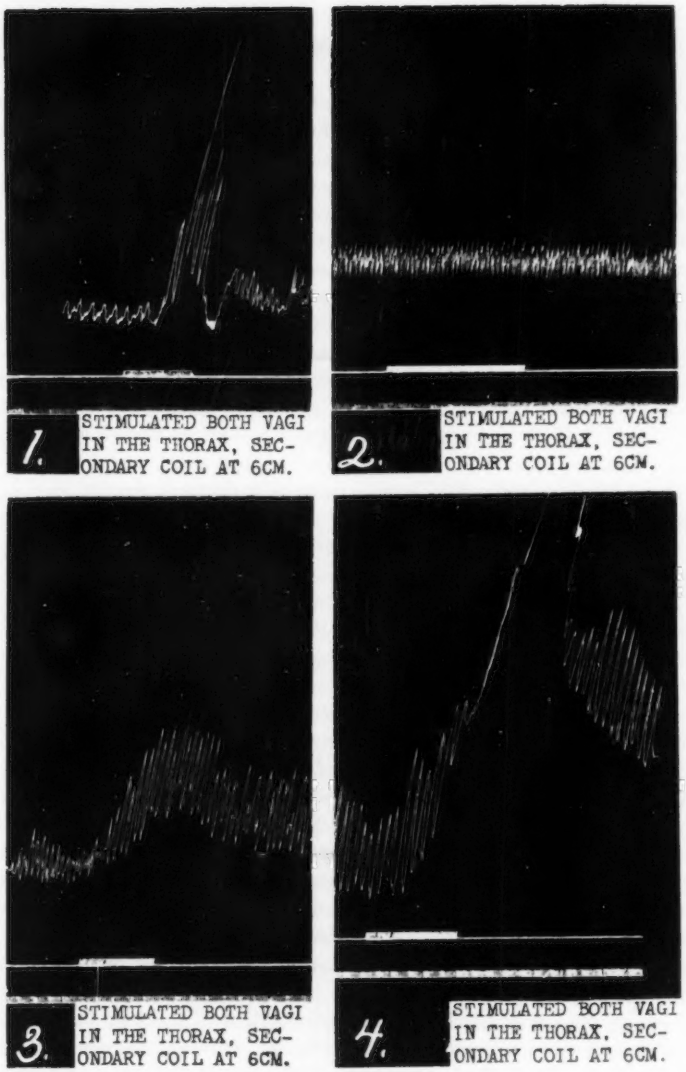

Fig. 1. Record showing the effect of increasing doses of nicotin on the response of the intestine to stimulation of both vagi below the level of the heart in a 7½ kilo dog.

1. Normal response before nicotin was given.

2. Complete absence of response 12 minutes after giving 2.5 mgm. of nicotin hydrochloride.

3. Recovery of response 4 minutes after giving 250 mgm. of nicotin hydrochloride. Total dose at this time 272.5 mgm.

4. Record obtained after a total dose of 5,522 mgm. of nicotin hydrochloride.

Upper line—contractions of the small intestine recorded with a piston recorder and balloon.

Middle line—signal in series with inductorium. Lower line—time in 1 second intervals.

RESULTS. Insofar as our procedure in a given experiment paralleled that of Bayliss and Starling, our results corroborate theirs; that is, we were able to confirm their observation that small doses of nicotin (1 to 20 mgm. in our experiments) bring about a condition of the intestinal vagus mechanism in which stimulation of the nerve is without effect on the muscle (tracing 2). This result was obtained in 7 out of 9 experiments. For convenience we will refer to this condition of the vagi as pseudoparalysis. In our experiments this pseudoparalysis of the vagi was generally associated with a more or less quiescent condition of the intestine. When larger doses of nicotin, e.g., 25 to 50 mgm. per kilo of either the akaloid or hydrochloride, were given to an animal in which pseudoparalysis of the vagi had been produced, stimulation of the vagus again became effective (tracing 3). In fact the responses of the intestinal muscle to vagus stimulation following the larger doses of nicotin frequently became more marked than they had been in the unpoisoned animal. Still larger doses, e.g., 50 to 500 mgm. per kilo, further augmented the response so that ultimately reactions to vagus stimulation were obtained which were much greater than any we observed in unpoisoned animals (tracing 4). Massive doses, e.g., 2000 to 3000 mgm. per kilo, finally caused paralysis of the intestinal vagi from which they did not recover during the period of observation.

The responses of the intestine to vagus stimulation following the administration of large doses of nicotin were qualitatively similar to those obtained before nicotin was given except in one respect. In the unpoisoned animal the motor response was frequently preceded by transient inhibition, whereas in the nicotinized animal the response was purely motor. In some of the records obtained after giving nicotin there was evidence of a slight decrease in the activity of the preparation just preceding the motor response but such occasional variations in the direction of inhibition were no greater than similar changes which appeared at irregular intervals without apparent cause. Therefore, we are inclined to consider these moderate reductions in activity as purely coincidental. However, it is possible that they represent a slight degree of inhibition that had not been abolished by nicotin.

In five of our experiments, the first stimulation of the vagi failed to influence intestinal motility in any way. In two of these, however, repeated stimulation finally elicited a response. The results of these two experiments are similar to those described by Bayliss and Starling as characteristic of their experiments. In the other three animals repeated stimulation failed to elicit any response until nicotin was given. Bayliss and Starling cited many such failures in their own earlier experiments as well as in the work of other investigators.

In our experiments, as in those of Bayliss and Starling, the usual response to vagus stimulation before the administration of nicotin was

primary inhibition both of the intestinal rhythm and tonus, followed by a motor response which consisted of an increase both in the tonus of the muscle and in the amplitude of the rhythmic contractions. The degree and duration of the inhibition, in our experiments, were variable and in two of the animals no inhibition was observed.

Discussion. In general the effects of nicotin have been described as consisting of stimulation when the dosage is small, followed by depression or paralysis, as the dosage is increased. The action of nicotin on the vagi has hitherto not been regarded as exceptional. In the case of the heart, for example, there is a primary decrease in rate indicating stimulation of the vagi followed by a secondary increase in rate generally interpreted as due to vagus paralysis (Langley and Dickinson, 1890). There are, so far as we know, no facts regarding the properties of nicotin which suggest an explanation of the peculiar results recorded in this paper. Furthermore, these results are difficult to explain by a pharmacological hypothesis because they constitute an apparent reversal of the almost universal mode of action of drugs, namely, stimulation with small doses followed by depression or paralysis when larger doses are given. Possibly the explanation is to be found in some peculiarity of the intestinal neuro-muscular mechanism rather than in a deviation in the case of nicotin from the usual mode of action of drugs.

It is not necessary to attribute every loss of function in a nervous mechanism to paralysis. Failure of response to nervous excitation may be caused not only by paralysis of some part of the conducting mechanism but also by inhibition. The result reported above resembles a type of inhibition involving the same mechanism described by Bayliss and Starling (1899). They say: "Among the conditions which may coöperate in preventing the motor effects of the vagus, considerable importance must be ascribed to anaesthetics and narcotics and the exposure and handling of the intestines with the circulatory changes thereby induced. More important than these factors are, however, the *inhibitory influences*" (Italics ours) "to which, as we have seen, all parts of the intestine are subject—influences which are partly reflex in origin and are started by stimulation of any sentient surface or the intestine itself, and transmitted through the splanchnic nerves, and influences which, originating in the intestine itself, tend through the local nervous mechanism to inhibit the activity of all the lower segments of the gut." They state further: "There is no doubt that the augmentor effects of vagal excitation are extremely susceptible to the influence of inhibitory stimuli. It is very difficult and in most cases impossible to evoke the augmentation" (by stimulation of the vagus) "unless the splanchnic nerves have been previously divided." Again, "It seems probable therefore, that the absence of the augmentor effect" (of vagus stimulation) "in a loop of intestine which has been ligatured above

or at both ends, is due not so much to the cutting off of the normal nerve channels as to the continued inhibition excited by the upper ligature." These quotations indicate clearly that the authors believed that the normal augmentor effects on the intestine of stimulation of the vagi could be kept in abeyance by inhibitory influences transmitted over the splanchnic nerves or through the local nervous mechanism of the intestine.

The work of Langley and Dickinson (1889) showed clearly that nicotin in appropriate amounts can act as an excitant to sympathetic nerves through an effect on the ganglia and later cause paralysis of the same structures. Bayliss and Starling observed temporary inhibition of the intestinal motor phenomena following small doses of nicotin. They attributed this inhibition to a stimulating effect of the nicotin on "the splanchnic fibers or their last cell station."

In view of the observations cited above the conclusion seems justified that 1, nicotin may cause excitation of the splanchnic (inhibitory) nerves to the intestine through an action on the ganglia, and that 2, excitation of the splanchnics may, by a process of inhibition, prevent the augmentor effects of vagus stimulation on the intestine. Therefore, it seems reasonable to suppose that nicotin, in appropriate amounts, could bring about loss of vagus function by a process of inhibition. However, inhibition of conduction in the vagus path to the intestine requires an appropriate functional arrangement of the vagus mechanism which includes the enteric plexuses.

Adopting the conception of inhibition and the terminology employed by Sherrington (1906), the conditions necessary for inhibition of conduction in a chain of efferent neurons may be said to be present only when some neuron in the chain is employed as a common path by impulses from more than one afferent neuron. In other words, inhibition, in the sense of the term as used in this discussion, can occur only at a synapse which transmits impulses from more than one source. In the present instance, the possibility of inhibition in the vagus path implies that the synapses between vagus fibers and their terminal neurons (neurons of the myenteric plexus) transmit impulses which reach them not only over the vagus but also over other neurons, for example, other neurons of the myenteric plexus or those making up the splanchnic nerves. Such a conception involves the assumption either that the myenteric plexus comprises local reflex arcs, or that the splanchnic fibers or their collaterals terminate in relation to neurons of the myenteric plexus. The possibility of such an arrangement has been suggested by Carlson (1922). As a "working hypothesis" he advanced the view that "the action of visceral efferents (sympathetic and autonomic), at least on some visceral motor mechanisms, are association or reflex responses and not simple peripheral responses like that of the skeletal muscles on stimulation of the pyramidal tracts. That is, the visceral efferent nerve fibers are in reality afferents to the local but diffuse reflex nervous centers in the visceral organs."

This hypothesis offers a ready explanation of the phenomenon which we are reporting as well as of the similar phenomenon of inhibition of vagus action observed by Bayliss and Starling. It might be assumed that the hypothetical "reflex" over the vagus is inhibited by initiating an antagonistic "reflex" over the splanchnic, just as one reflex inhibits another in the spinal cord. However, such an interpretation is beset with certain difficulties. Langley (1896) and also Bunch (1898) observed that the postganglionic sympathetic fibers to the intestine are not paralyzed by moderate doses of nicotin. They concluded, therefore, that the splanchnic efferent chains to the intestine comprise but one synapse and that this synapse is located outside the intestinal wall. This conclusion, which also implies that the splanchnic efferent chains are not functionally related to the enteric plexuses but terminate only in the gastro-intestinal musculature, has not been successfuly controverted. According to this view the peripheral vagus and splanchnic mechanisms are quite independent of each other. There is, therefore, no secure ground for the assumption that vagus intestinal reflexes are inhibited by reflexes carried out over splanchnic reflex arcs.

On the other hand the hypothesis that the visceral efferent fibers are in reality afferents to the local reflex centers in the visceral organs may be applied to the gastro-intestinal vagus efferent components without difficulty. According to current teaching vagus efferent neurons are intercalated between the central nervous system and the myenteric plexus. If the myenteric plexus may be regarded as comprising local reflex centers, the vagi may well be regarded as constituting association pathways between these centers and the central nervous system. Limited in this manner, Carlson's hypothesis might still explain inhibition of conduction over the vagi. It might be assumed that the efferent limb of a local reflex arc constitutes the common path both for local and vagus reflexes. The two reflexes, as in comparable instances in the central nervous system, would be mutually exclusive in the use of the common path. A local reflex stimulated by drug action or manipulation of the intestine might thus inhibit conduction over the vagi. This view, however, affords no basis for the explanation of inhibition of vagus conduction by splanchnic stimulation described by Bayliss and Starling, unless we modify the view held by Langley and by Bunch, to the extent of admitting that collaterals of the splanchnic fibers may reach the myenteric plexus.

In another respect, the results of the present study fail to conform to current pharmacological and physiological teaching. It is generally believed that the paralytic effect of nicotin involves both sympathetic and parasympathetic ganglia. According to current teaching the ganglia of the myenteric plexus are parasympathetic ganglia in the vagus path to the stomach and intestine. The results obtained by Bayliss and Starling

in their study of the effect of nicotin on the vagus mechanism would lead one to believe that the effect of nicotin is the same on these parasympathetic ganglia as on other autonomic ganglia. Our observation that doses of nicotin hundreds of times larger than the doses generally regarded as sufficient to block conduction in other autonomic pathways does not abolish conduction over the vagus path to the intestine, is difficult to reconcile with such a view. Apparently either some of the vagus fibers reach the intestinal muscle without making synapse in the myenteric and submucous plexuses or these plexuses differ from other ganglia of the autonomic system in their reaction to nicotin.

With reference to the inhibition which precedes the motor effect of vagus stimulation, the fact that nicotin in large doses causes a loss of this primary inhibitory effect also requires explanation. If we take the view that the vagus nerve actually contains inhibitory as well as motor fibers to the intestinal muscle we must suppose that the inhibitory mechanism is more susceptible than the motor mechanism to the action of nicotin. There is reason to believe that nicotin in fairly large doses has a tendency to free the intestinal muscle from inhibitory influences, for after such doses the rhythmic activities of the muscle are tremendously augmented. On the other hand it is possible that the inhibitory effect of vagus stimulation may be only a manifestation of the activity of the myenteric reflex (law of the intestine) and is associated with motor activities occurring at higher levels. The experiment described by Bayliss and Starling in which primary inhibition was obtained in the first part of the duodenum by stimulation of the vagus does not exclude this possibility. Such inhibition might be due to reflexes from a contracted stomach. See Joseph and Meltzer (1910), Wheelon and Thomas (1922), and Alvarez and Mahoney (1923).

Finally in so far as the results of the present series of experiments are in harmony with any particular view hitherto advanced, they lend support to the theory that the enteric plexuses are reflex centers, manifesting such complex phenomena as inhibition of reflexes, and that the vagus nerves function as association paths between central and peripheral reflex centers rather than as simple efferent pathways.

SUMMARY

The observation by Bayliss and Starling that small doses of nicotin cause a loss of the effects of vagus stimulation on the small intestine in dogs is confirmed. However, if nicotin is given in gradually increasing doses the loss of vagus function is only temporary and is fully reëstablished when 25 to 50 mgm. per kilo have been administered. After still larger doses the response of the intestine to vagus stimulation becomes greater than before nicotin was given.

The view is proposed that the temporary loss of vagus function is not due to paralysis of the vagus mechanism but is a manifestation of inhibition. The possible functional arrangements of the intestinal vagus mechanism permitting of such inhibitory phenomena are discussed. The arrangement which appears to be most in harmony with the available data comprises local reflex arcs in the myenteric plexus, with the vagus fibers acting as association pathways between the central nervous system and local reflex centers in the intestine.

Purely motor effects on the intestine are obtained from vagus stimulation following large doses of nicotin, whereas before nicotin is given the motor effect is usually preceded by transient inhibition. This result indicates that the mechanism responsible for the primary inhibitory effect is rendered non-functional by the action of nicotin. This mechanism may either comprise inhibitory fibers in the vagus nerve, or the arcs responsible for the myenteric reflex.

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A STUDY OF GASTRO-INTESTINAL MOTILITY IN RELATION TO THE ENTERIC NERVOUS SYSTEM

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According to the current anatomical teaching, the autonomic nervous system is essentially an efferent system; i.e., all its neurons are post-ganglionic neurons in visceral efferent chains. According to this view, the only afferent neurons associated with the visceral organs are the visceral afferent components of the cerebrospinal nerves. This conception, applied to the nervous plexuses located within the walls of the digestive tube, is incompatible with many physiological observations which seem to indicate the presence of an enteric nervous mechanism through which coördinated reflexes are carried out. A recent anatomical investigation by one of the present writers (Kuntz, 1922) has also revealed synaptic relationships between neurons in the enteric plexuses which indicate the occurrence of enteric reflex arcs.

Any adequate interpretation of physiological phenomena involving a nervous mechanism must be compatible with the facts regarding the anatomical structure of that mechanism. On the other hand, physiological phenomena may be important criteria in the interpretation of anatomical data. The present investigation was undertaken in order to obtain further data regarding the functional relationships of the enteric nervous system and its rôle in gastric and intestinal motility. The data obtained also have a bearing on the genesis of the rhythmic contractions in the stomach and intestine.

GASTRO-INTESTINAL MOTILITY. The principal types of gastro-intestinal motility are peristalsis and hunger contractions in the stomach, peristalsis and rhythmic segmenting contractions in the small intestine and peristalsis and anti-peristalsis in the large intestine. Gastro-intestinal motility of all these types has been observed following section of the extrinsic nerves supplying the stomach and intestine. Consequently, it is generally assumed that this motility, though normally subject to central nervous control through the extrinsic nerves, originates in the neuromuscular mechanism in the walls of the stomach and intestine. Whether motility of certain of these types is initiated in the enteric nervous system or in the enteric musculature must be regarded as an unsettled question.

REVIEW OF LITERATURE. 1. *Data bearing on the occurrence of enteric reflexes. a. Stomach.* Cannon (1906) observed that the difference in the rate of discharge of different kinds of food from the stomach persists following section of both splanchnic and both vagus nerves. He also observed that relaxation of the pyloric sphincter when the contents of the pyloric antrum become acid occurs also in the excised stomach (Cannon, 1907). He also described tonic contractions of the cardiac sphincter when the contents of the stomach became acid (Cannon, 1908). This phenomenon occurred following section of both vagi and destruction of the thoracic and lumbar portions of the spinal cord. He concluded that it is likewise a reflex phenomenon carried out through the enteric plexuses.

Carlson (1913) observed that hunger contractions of the stomach are inhibited when certain substances are introduced into this organ. Carlson and Brunheimer (1914) observed inhibition of the hunger contractions of the empty stomach when the same substances were placed in the intestine. This phenomenon also occurs following section of the extrinsic nerves. Carlson's diagram (1913) illustrating the nervous mechanism involved in the reaction indicates clearly that he regards it as a local reflex phenomenon which involves both afferent and efferent neurons.

Luckhardt, Phillips and Carlson (1919) observed that mechanical irritation of the duodenal mucosa, through a duodenal fistula, in dogs with extrinsic nerves to the stomach and intestine intact, elicits tonic contraction of the pyloric sphincter. We observed the same phenomenon in dogs following section of both vagi and both splanchnic nerves. However, the same reaction could not be elicited when conduction through the local neuromuscular mechanism was arrested by compression of the wall of the proximal portion of the duodenum between a ligature on the outside and a solid cylindrical body in the lumen.

b. Small intestine. Bayliss and Starling (1899, 1900b) observed that stimulation of the small intestine usually results in contraction of the musculature above and relaxation below the point at which the stimulus is applied. A bolus in the lumen of the intestine is the most efficient stimulus for this reaction. It also occurs following section of the extrinsic nerves to the intestine, but is abolished by nicotin and certain other drugs when administered in doses which Bayliss and Starling regarded as sufficient to paralyze the enteric nervous mechanism. They observed that contractions initiated by the bolus tend to advance along the intestine and to displace the bolus aborally. They concluded that peristalsis in the small intestine is a local reflex phenomenon.

Henderson (1923) recently observed that peristaltic contractions initiated by increasing the internal pressure in the small intestine cease, apparently by reason of fatigue, while the muscle is still irritable and able to contract strongly in response to direct stimulation.

Cannon (1912) observed that an incision around the small intestine which divides both the longitudinal and circular muscle and the myenteric plexus interrupts peristaltic waves. He also observed in excised pieces of the esophagus, stomach, small and large intestine, the reflex described by Bayliss and Starling and designated it the "myenteric reflex."

King and Arnold (1922), in a study of the motor activities of the muscularis mucosae and the intestinal villi, observed retraction of the villi and ridging and pitting of the mucosa in response to mechanical and chemical stimuli applied to the intestinal epithelium. These phenomena also occur following degeneration of the splanchnic nerves. Inasmuch as stimulation of the vagi has no apparent effect on the motility of the intestinal villi these authors concluded that vagus fibers do not reach the muscularis mucosae. They interpreted the movements of the mucosa and intestinal villi as reactions which are mediated through the local neuromuscular mechanism.

c. Large intestine. Bayliss and Starling (1900a), Elliott and Barclay-Smith (1904) and Langley and Magnus (1905), after studying the denervated colon, all concluded that peristalsis in the large intestine involves a local reflex mechanism similar to that which governs peristalsis in the small intestine. Lyman (1913) observed, in animals in which the spinal cord had been destroyed, that anti-peristalsis in the colon ceases when food enters it from the ileum.

2. *Data bearing on the genesis of certain rhythmic gastro-intestinal contractions.* Bayliss and Starling (1899) observed that rhythmic contractions in the small intestine persist following the administration of drugs in doses which they regarded as sufficient to paralyze the myenteric plexus. Under these conditions they also observed waves of contraction, which were not, like peristaltic contractions, preceded by inhibition, advancing indifferently in either direction along the small intestine. Elliott and Barclay-Smith (1904) observed that anti-peristalsis in the large intestine persists following the administration of nicotin in doses sufficient to abolish intestinal peristalsis. Cannon (1909) observed that gastric peristalsis is not abolished by "large doses of nicotin" (dosage not stated) nor by multiple incisions through the muscular layers of the stomach which he regarded as sufficient to eliminate the functional activity of the myenteric plexus (Cannon, 1911). He also observed that multiple incisions through the muscular layers of the small intestine do not abolish rhythmic contractions (Cannon, 1912).

In view of the results of their experimental work with nicotin and other drugs, Bayliss and Starling (1899) advanced the theory that the rhythmic contractions of the intestine, i.e., those which persist after the coördinated or reflex peristaltic movements are abolished, are myogenic. This conclusion may be essentially correct though it is not fully substantiated by

the results of their experimental work with nicotin. Their inference that all function in the enteric plexuses disappears with the abolition of the myenteric reflex has not been universally accepted. King and Arnold (1922) found that the responses of the intestinal villi to chemical and mechanical stimulation are not abolished by nicotin until enough has been given to paralyze the muscularis mucosae. They interpreted these reactions as reflex activities of the submucous plexus. They seem to believe that nicotin does not affect this plexus in the same way that it is said to affect other autonomic ganglia. Neither are they convinced that it paralyzes the myenteric plexus. We have shown in another communication (Thomas and Kuntz, 1926) that the influence of the vagi on the small intestine is not abolished, as judged by the motor effects of vagus stimulation, by doses of nicotin many times as large as those used by Bayliss and Starling to abolish the peristaltic reflex. The dose which they used (2 or 3 cc. of 1 per cent solution for a small dog) if not increased, prevents the manifestation of the characteristic effects of vagus stimulation. However, when the dose is greatly increased the vagus function returns and persists until a quantity of nicotin hydrochloride representing 2 to 3 grams of the undiluted alkaloid per kilo has been administered. Efferent vagus fibers do not terminate directly on muscle cells but in synaptic relationship with enteric neurons, and therefore the integrity of the efferent vagus chain depends upon the integrity of some of the synapses in the myenteric plexus. Obviously, the small dose of nicotin used by Bayliss and Starling does not paralyze the nervous mechanism but merely prevents the manifestation of certain of its functions, possibly by a process of inhibition. Consequently, the experimental results reported by Bayliss and Starling do not prove that the rhythmic contractions which persist after the myenteric reflex has been abolished by nicotin are not carried out through reflex arcs.

The experimental results recorded by Magnus (1905), Gunn and Underhill (1914), and Alvarez and Mahoney (1922) are somewhat more convincing. These investigators took advantage of the fact that the longitudinal muscle, with the myenteric plexus adhering to it, may be separated from the circular muscle. Thus, circular muscle may be obtained practically free from nervous elements, especially if only the deeper layers are used. They observed rhythmic contractions in strips of intestinal muscle denervated in this manner, although Magnus failed to obtain them except after the use of stimulating drugs. These results indicate that, under certain conditions, the intestinal muscle is capable of rhythmic contractions in the absence of nervous influences. They do not prove the muscular origin of rhythmic movements of the stomach and intestine in the intact animal.

In view of the observations cited above the classification of the motor activities of the small intestine proposed by Bayliss and Starling may be

extended to include practically all types of gastro-intestinal motility. This classification may be summarized as follows: 1, movements which are obviously reflex, including peristalsis both in the large and small intestine, and many other coördinated contractions; 2, rhythmic contractions, including gastric peristalsis, segmenting contractions in the small intestine, and anti-peristalsis and other rhythmic contractions in the large intestine.

EXPERIMENTAL METHODS. *a. Pharmacologic data on which experimental methods are based.* The experimental procedures employed in this study are based on certain facts regarding the pharmacology of nicotin which are still unpublished. They will therefore be briefly reported here.

Langley and Dickinson (1890) called attention to a curare-like action of nicotin on skeletomotor nerve endings. This fact suggested to us the possibility of demonstrating a similar reaction of the nerve endings in the involuntary muscle of the gut. Nicotin in concentrations of 1 part in 500 of Locke's solution applied to excised pieces of intestine, or in doses of 2 or 3 grams of nicotin hydrochloride per kilo administered to intact animals (dogs), was found to be sufficient to completely paralyze the nervous mechanism. The evidence at present available which indicates that complete paralysis may be produced in certain peripheral elements of the nerve supply to the gastric and intestinal musculature, may be summarized as follows: Excised pieces of intestine with the mesenteric blood vessels and nerves attached, immersed in oxygenated Locke's solution, contract rhythmically and respond in a characteristic manner to electrical stimulation of the attached nerves. The addition of nicotin in the concentration mentioned above completely abolishes all responses to stimulation of the nerves, some of the fibers of which are post-ganglionic sympathetic fibers. Replacing the nicotin with Locke's solution restores the capacity to respond to nervous stimulation. This shows that the loss of irritability was due to the nicotin and not to fatigue or other causes, such as over-stimulation. Identical results were obtained with the intestine of dogs, cats and rabbits when recording contractions either of the longitudinal or the circular muscle. When the contractions of the intact circular muscle of the small intestine, in anesthetized dogs, were recorded by the balloon method, stimulation of the mesenteric nerves supplying the part under observation elicited characteristic responses (fig. 2, *A*). The administration of nicotin in the dosage mentioned, abolished all responses to electrical stimulation of the mesenteric nerves (including some post-ganglionic sympathetic fibers) (fig. 2, *B*). When no further nicotin was given, the nervous mechanism gradually recovered its conductivity (fig. 2, *C*).

Abolition of responses to nerve stimulation, both in the excised strips and the intact intestine, is accompanied by loss of the normal responses to adrenalin and apparently also to pilocarpin. In excised strips, particu-

larly after the nicotin had been allowed to act for an hour or longer, the loss of responsiveness to adrenalin was so complete that massive doses of it had no effect either on the tonus or the rhythmic contractions (fig. 1, *A*). In the intact animal the loss of responsiveness to massive doses of adrenalin

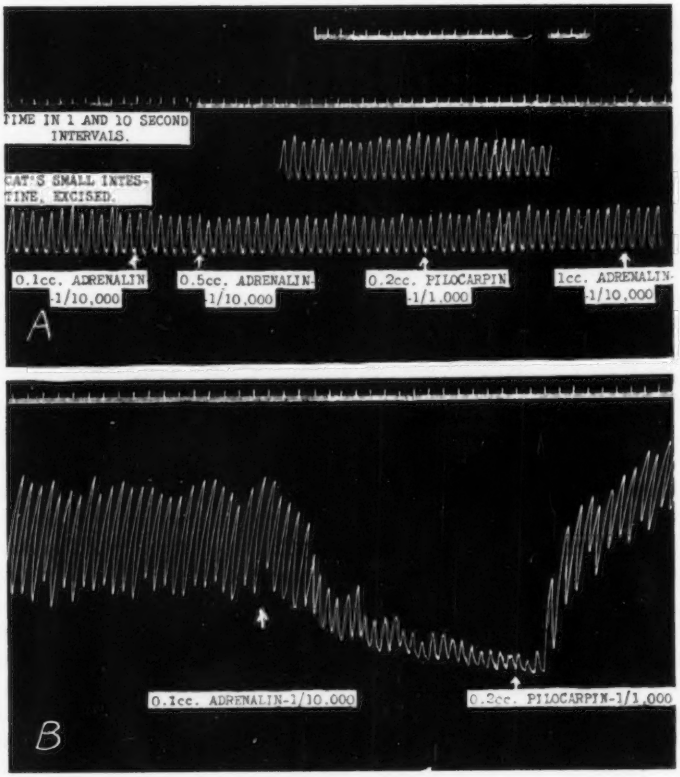

Fig. 1. Record showing the effect of nicotin on the rhythm and response to adrenalin and pilocarpin of the longitudinal muscle of the excised ileum (cat).

A. Preparation immersed in 0.2 per cent nicotin hydrochloride in Locke's solution.

B. Control record from the same preparation after washing away the nicotin and immersing in plain Locke's solution.

Time originally in 1-second intervals. Retouched to show 10-second intervals. Volume of bath, 25 cc.

was less complete, but moderate or even large doses (0.5 cc. of 1/1000 solution for a 6 kilo dog) produced no effect either on the intestinal muscle or the blood pressure. Abolition of the reaction to pilocarpin has been

investigated in but few experiments and on the excised intestine only. However, in every case in which this drug was applied after the muscle no longer responded to adrenalin, moderate concentrations (0.008 mgm. per cc.) had no effect and high concentrations (0.1 mgm. per cc.) abolished the rhythmic contractions. We concluded, on the basis of this evidence, that nicotin in the dosage mentioned completely paralyzes the nervous mechanism and that intestinal muscle so treated might be regarded as even more completely denervated than that which is anatomically separated from the plexus, since in the latter case there still remain terminal nerve filaments and nerve endings. The exact site of this action of nicotin has not as yet been determined, but it appears to be located as far peripherally as the site of the action of adrenalin and pilocarpin.

The effect of denervation by massive doses of nicotin was then studied on the longitudinal and circular muscle in the excised small intestine of dogs, cats and rabbits and in the stomach and small intestine, in situ, in dogs.

b. Excised intestine. The rabbit's intestine was obtained from animals killed by a blow on the head; the intestine of dogs and cats from animals killed by section of the medulla or from animals anesthetized with ether. In all cases a piece of the small intestine, usually the duodenum or upper jejunum, was removed immediately after death and its contents washed out at once by running Locke's solution through it under pressure. It was then placed in iced Locke's solution and kept in the refrigerator until used.

The usual methods of recording the rhythmic contractions of the excised intestine were employed. To obtain records of the contractions of the longitudinal muscle, pieces 4 or 5 cm. in length were cut off and suspended between a bent rod and the short arm of a heart lever by a loop of thread passed through the wall at each end. The tension was just sufficient to extend the piece fully when immersed but not sufficient to support its weight when suspended in air. The pieces were immersed in Locke's solution warmed to 38°C. in a constant temperature water bath. Oxygen was kept bubbling freely through the solution at all times. To obtain records of the contractions of the circular muscle, pieces of the same length were used, and other conditions were the same except that the loops of thread were attached at the sides instead of at the ends of the piece. The loops were passed through the muscle layers at points as nearly as possible opposite each other and equally distant from both ends. In this manner records were obtained of the contractions of a ring of circular muscle some distance from any injured part of the intestine.

No difficulty was experienced in obtaining vigorous rhythmic contractions either of the longitudinal or circular muscle of the intestine of the rabbit and the cat. Both longitudinal and circular muscles of the dog's

intestine were slow to begin and contracted only feebly and irregularly. Possibly this was due to the greater mass of the intestine and the consequent inefficient exchange of oxygen and metabolic products between the muscle and the bath.

The bath of Locke's solution in which the piece of intestine was immersed was always of known volume (generally 200 cc.). When denervation was desired, a 10 per cent solution of nicotin in Locke's solution neutralized with hydrochloric acid to the pH of the bath was added in measured quantities.

c. Stomach and intestine in situ. To obtain records of the contractions of the intestine in situ, dogs were anesthetized with ether, both splanchnics and both vagi cut, the abdomen opened by a midline incision and a balloon placed in a convenient loop of small intestine through an incision below the ultimate position of the balloon. The balloons were made by tying the closed end of a rubber condom over the tip of a soft rubber catheter. During the experiment the abdomen was kept closed except when necessary to open it for operative procedures or nerve stimulation. The contractions of the stomach were recorded by a double balloon fixed in the antrum and pyloric canal according to the technic described by Wheelon and Thomas (1922). In all cases pressure was maintained in the balloon by means of a water manometer. The pressure was varied from time to time and maintained at the level which seemed to give the best results with the viscus under observation. The pressure used ranged from 5 to 20 cm. of water. Somewhat higher pressures were used in the stomach balloons than in the intestinal balloons. Records were made either with piston or bellows recorders. The nicotin solution employed was the same as that used in the experiments with excised intestine, namely, 10 per cent of the hydrochloride. It was permitted to flow from a burette into the femoral vein. The drug was administered in divided doses. The initial dose was usually 1 or 2 cc. of the solution. Later individual doses were increased up to 25 or 50 cc.

EXPERIMENTAL RESULTS. 1. *Gastro-intestinal movements which persist following paralysis of the enteric nervous system with nicotin. a. In excised pieces of the small intestine.* The intestine of the cat was most favorable for the study of excised segments in the presence of large quantities of nicotin. Satisfactory evidence of paralysis of the nervous mechanism by means of nicotin was obtained in fifteen preparations of cat's intestine. In five of these the reaction of the muscle to stimulation of the mesenteric nerves alone, in eight the reactions of the muscle to adrenalin alone, and in two the reactions of the muscle both to adrenalin and nerve stimulation were studied. Approximately the same concentration of nicotin (2 mgm. per cc. of immersion fluid) was required to abolish responses to nerve stimulation as was required to abolish the reaction to adrenalin, but a

comparison of the experiments by the two methods suggested that the response to nerve stimulation is abolished somewhat more promptly than the reaction to adrenalin. This difference was evident in both experiments in which both adrenalin and nerve stimulation were employed. Good rhythmic contractions continued in all the preparations of cat's intestine after the tests used to determine the functional state of the nervous mechanism failed to elicit any further response (fig. 1 *A*).

Rhythmic contractions usually subsided, in the rabbit's intestine, in the presence of any considerable amount of nicotin. The muscle either lost tonus and became quiescent or it tended to go into tetanus and failed to relax rhythmically. The rhythmic contractions subsided while the preparation was still able to respond in a characteristic manner to adrenalin and nerve stimulation, consequently the cessation of rhythmic contraction could not be regarded as the result of paralysis of the nervous mechanism. In one preparation of rabbit's intestine rhythmic contractions continued after the reaction to adrenalin could no longer be elicited. In this preparation the muscle had gone into tetanus following a moderate dose of nicotin and was relaxed by the addition of adrenalin, after which rhythmic contraction was resumed and continued even after the addition of the usual paralytic dose of nicotin (2 mgm. per cc. of bath).

As stated above, the excised intestine of the dog was not particularly active. However, rhythmic contractions continued in two of our preparations after both adrenalin and nerve stimulation no longer elicited any response.

b. Intact small intestine, stomach and large intestine. Satisfactory evidence of paralysis of the nervous mechanism in the intact small intestine of the dog was obtained in four experiments. In two of these experiments stimulation of the mesenteric nerves no longer elicited any response. In one of these the reaction to adrenalin was also abolished. Doses of 0.25 mgm. and 0.5 mgm. of adrenalin given intravenously in succession (7.5 kilo dog) brought about no relaxation of the muscle. In the other two experiments the evidence of nicotin paralysis was not quite so complete but the reaction to stimulation of the mesenteric nerve-fibers was reduced to such an extent that it was barely apparent in the record. The reaction was no greater than could be obtained by placing the electrodes on other tissues as near the muscle, the contractions of which were being recorded, as the point on the nerve selected for stimulation. The residual reaction in these cases was probably the result of direct stimulation of the muscle due to the spread of current. In all four experiments the rhythmic contractions were not abolished by the nicotin but continued, after paralysis of the nervous mechanism, with greater amplitude than in the beginning of the experiment (fig. 2 *B*).

The experimental results obtained in the study of gastric peristalsis

could not be controlled as satisfactorily as those involving contractions of the small intestine because the post-ganglionic nerve fibers leading to the stomach are not easily accessible. It was, therefore, necessary to rely on the reaction to adrenalin, which is not altogether clear-cut in the intact animal. In some instances, in deeply nicotinized animals, adrenalin caused retardation and weakening of the heart, in others it caused fibrillation. We could not be certain whether the reduction in amplitude in some of our records of gastric contractions following the injection of adrenalin was due to the characteristic action of adrenalin on nerve endings in the stomach or to further weakening of the already feeble circulation. However, we succeeded in two animals in producing with nicotin a condition

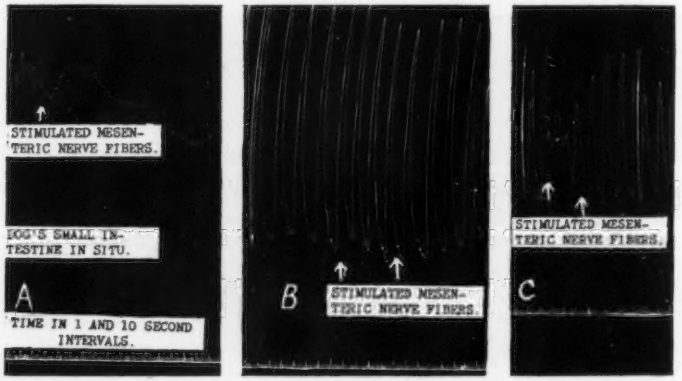

Fig. 2. Record showing the effect of nicotin on the rhythm and responsiveness to stimulation of the mesenteric nerve fibers of the dog's small intestine in situ.

A. After 75 mgm. of nicotin per kilo.

B. After 3,262 mgm. of nicotin per kilo.

C. After an attempt to eliminate nicotin effect by giving Locke's solution intravenously.

Time originally in 1-second intervals. Retouched to show 10-second intervals.

in which moderate doses (0.1 mgm.) of adrenalin no longer had any effect on the stomach (fig. 3 *B*). In several animals larger doses of nicotin than were required to abolish responses to stimulation of the post-ganglionic sympathetics to the small intestine in other experiments did not completely eliminate the effects of adrenalin on the gastric musculature. In view of the results obtained in the experiments on the small intestine, it seems probable that the local nervous mechanism was paralyzed in the experiments on the stomach also. At any rate, its irritability was markedly reduced. The rhythmic activity of the stomach was not abolished in any of these experiments. (See fig. 3 *B*.) Indeed gastric contractions

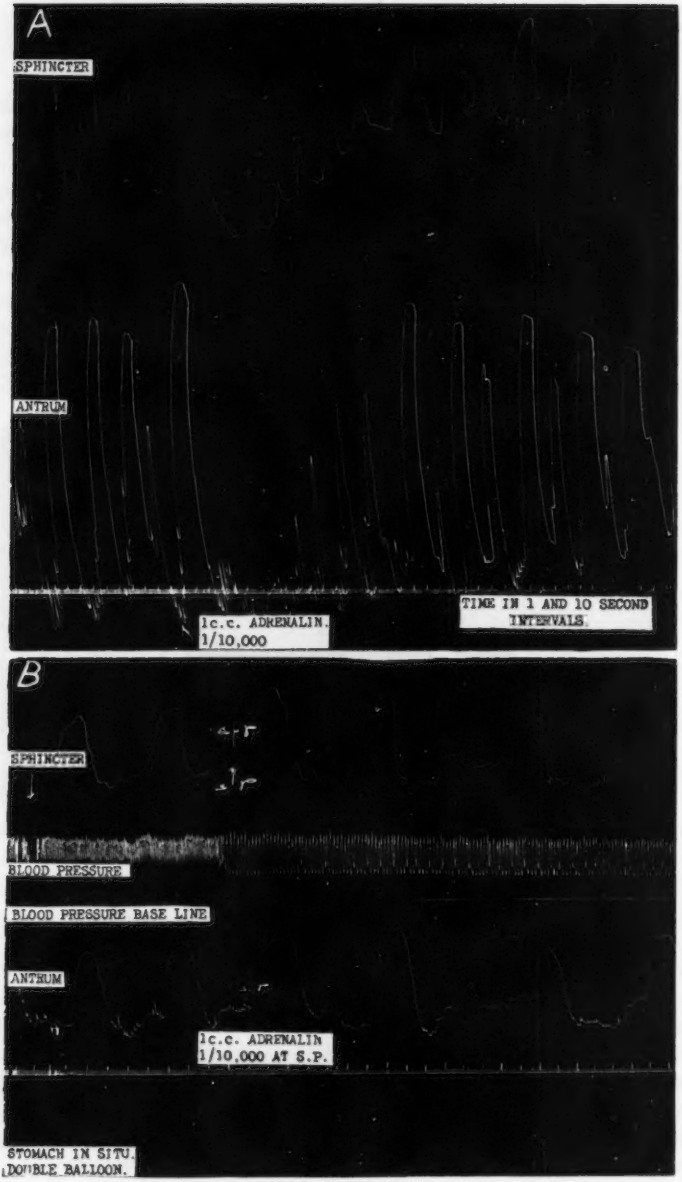

Fig. 3. Influence of nicotin on the rhythm and response to adrenalin of the stomach in situ in a 4.5 kilo dog.

A. After 4.1 grams of nicotin hydrochloride had been administered.

B. Record obtained from the same preparation after 9.6 grams of nicotin hydrochloride had been administered. Between *A* and *B* a blood pressure record was begun.

Time originally in 1-second intervals. Retouched to show 10-second intervals.

continued for some time after the heart was stopped by still larger doses of nicotin. On direct inspection of the exposed stomach the rhythmic contractions which persisted were seen to progress over the viscus in essentially the manner of normal peristaltic waves.

The large intestine was studied in but one dog. After administration of nicotin in doses sufficient to abolish responses to stimulation of postganglionic nerve fibers to the small intestine, anti-peristaltic movements were observed in the exposed colon. These contractions continued to follow one another at regular intervals until the experiment was terminated about thirty minutes after the viscus was first observed. As stated above, Elliott and Barclay-Smith observed anti-peristaltic contractions in the colon in some experimental animals after peristalsis was abolished by nicotin. However, they deny the occurrence of anti-peristalsis in the large intestine of the dog. Colonic anti-peristalsis was unmistakable in our experiment. We have no data regarding its occurrence in dogs under normal conditions. Contractions were also seen in the small intestine which appeared to progress indifferently either forward or backward. This phenomenon, as stated above, was observed by Bayliss and Starling (1899) following moderate doses of nicotin sufficient to eliminate the myenteric reflex.

2. *Changes in the character of the rhythmic contractions in the small intestine following denervation with nicotin.* Nicotin in doses sufficient to paralyze the nervous mechanism produced essentially the same ultimate results in all of our preparations regardless of whether the viscus was excised or studied in situ. In all cases virtually all irregularities in the activity of the preparation were abolished. This change from the normal rhythmic activity, which is always more or less irregular, to the perfectly regular rhythmic activity of the nicotinized preparations seems to be correlated with the loss of function in the nervous mechanism. As long as a definite irregularity persisted, stimulation of the mesenteric nerves or the administration of adrenalin elicited some response (figs. 1 *B* and 2 *A*). On the other hand, when the perfect regularity characteristic of the nicotinized preparations was fully established neither nerve stimulation nor adrenalin had any effect (figs. 1 *A* and 2 *B*).

An attempt was made to analyze some of the irregularities in the records obtained before paralysis of the nervous mechanism, in the hope of obtaining further information regarding the functions of the enteric plexuses. The irregularities seen on the tracings may be classified as 1, changes in tonus; 2, changes in the amplitude of the rhythmic contractions; and 3, apparent changes in the rate or frequency of the contractions. Changes in tonus may occur spontaneously both in excised segments and in the intestine in situ. True changes in tonus are probably less frequent than irregularities which appear on the records as changes in tonus but are due

to other influences. For example, a wave of contraction passing over a segment of the intestine which is writing a record of its rhythmic contractions will produce a change in the record which looks like a temporary increase in tonus. Many of the apparent changes in the tonus of excised segments are the result of such progressing contractions which travel from one end of the segment to the other. Another cause of apparent changes in tonus is interference between rhythmic contractions of adjacent segments. The recording apparatus frequently is affected by the contractions of more than one segment of muscle. These segments apparently may contract at slightly different rates so that at times they reinforce each other in their effects on the recording lever and at other times they interfere so that the writing lever is scarcely moved. Such interference influences not only the height to which the lever is raised by the contraction but also the extent to which it is permitted to fall by the succeeding relaxation and thus produces an apparent change in tonus, as well as in the amplitude of the contractions. Similar phenomena of interference in the records of rhythmic contractions of the small intestine were observed by Bayliss and Starling (1899).

The rate of the rhythmic contractions in excised pieces of intestine remained fairly constant, in any given experiment, until nicotin was present in relatively high concentration. Nerve stimulation or administration of adrenalin or nicotin in small amounts usually had no effect on the rate of the rhythmic contractions. Occasionally an increase or decrease of one or two contractions per minute was noted. Such changes are probably associated with experimental manipulations. Since they did not occur regularly we do not regard them as characteristic results of the experiments.

In the records of the intestinal contractions obtained by the balloon method in intact animals, the rate appears on superficial examination to be more variable. Careful analysis of the records, however, revealed the fact that all the rates observed in any single experiment sustain a simple numerical ratio to each other. The higher rates approximate twice or three times the lowest rate observed. The higher rates were probably due to the action of more than one segment of the intestine on the balloon. Nicotin in large doses does affect the rate as well as the amplitude and regularity of the rhythmic contractions. In all of our experiments, both on intact and excised preparations, the rate of the rhythmic contractions was reduced progressively as the paralysis of the enteric nervous mechanism approached completeness.

Disregarding the effects of small doses and the early effects of large doses of nicotin, which are very complex, the amplitude of the contractions was first increased and then reduced by the progressive administration of nicotin. Larger doses than were required to paralyze the nervous mechanism finally stopped all rhythmic contractions.

DISCUSSION. *a. Morphology of the enteric nervous system.* The occurrence of coördinated reflexes in the stomach and intestine following section of the vagus and splanchnic nerves, as observed by earlier investigators, constitutes a physiological demonstration of the functional activity of an enteric reflex mechanism. The manner in which the enteric nervous system functions in the rhythmic contractions of the musculature of the stomach and intestine is less obvious. Studies of the rhythmic gastro-intestinal activities have led certain investigators, notably Alvarez (1922), to conclude that the enteric nervous system is essentially a nerve net involving actual protoplasmic continuity between the neurons. Erick Müller (1921) also described the enteric plexuses, in birds and mammals, anatomically as consisting in part of nerve nets and in part of free neurons. His findings were discussed in an earlier paper (Kuntz, 1922) and need not be set forth more fully at this time. Kuntz's anatomical preparations afford no evidence of the occurrence of nerve nets in the enteric plexuses in cats and dogs. Neither did Johnson (1925), in his recent anatomical study of these plexuses in cats and dogs, find evidence of protoplasmic continuity between neurons.

The theory that the enteric plexuses comprise local reflex arcs through which coördinated reflexes are carried out is not new. However, it has not been generally accepted either by anatomists or physiologists. Furthermore, we know of no anatomical demonstration of the occurrence of enteric reflex arcs preceding the investigation referred to above (Kuntz, 1922). Therefore, the essential facts regarding our knowledge of the anatomical structure of the enteric nervous mechanism, including the findings just referred to, will be briefly set forth.

The enteric nervous system comprises the myenteric plexus, the ganglia of which are located between the longitudinal and circular muscle layers, and the submucous plexus, the ganglia of which are located in the submucosa. The ganglia in both plexuses are connected with each other by commissures of non-medullated fibers. Fibers may be traced from the ganglia in the myenteric plexus into the longitudinal and circular muscle layers where they terminate on muscle fibers, and, through commissures, into the submucous plexus. Likewise, fibers may be traced from the submucous plexus, through the same commissures, into the myenteric plexus. Fibers may also be traced from the submucous plexus into the muscularis mucosae, among the gastric and intestinal glands and into the gastric and intestinal epithelium.

Pre-ganglionic vagus fibers terminate in synaptic relationship with enteric neurons. The pre-ganglionic fibers in the splanchnic nerves terminate in the coeliac and other prevertebral plexuses. According to the current teaching, the post-ganglionic fibers which take origin in the coeliac plexus and enter the walls of the stomach and intestine do not make

synaptic contact with enteric neurons, but terminate on the gastrointestinal musculature.

In the anatomical studies referred to above (Kuntz, 1922) synapses were described which involve two enteric neurons. These are quite independent of the intercellular network of fine fibers in the ganglia. They can be observed, in our preparations, only in instances in which the axon with its terminal arborization is stained intensely and the neuron in relation to which it terminates is stained lightly. Such synapses occur both in the myenteric and submucous plexuses. Some of them involve two neurons in the same ganglion. In such instances, in favorable preparations, the axon of the one may be traced without interruption from its origin to its termination by arborization around the cell body of the other neuron. Other synapses involve fibers which enter the ganglia through commissures. Some of these may be pre-ganglionic vagus fibers. Others are the axons of enteric neurons. Some of these approach the myenteric ganglia through commissures leading from the submucous plexus. These are probably the axons of neurons in the submucous plexus. Synapses involving fibers which arise in the myenteric and terminate in the submucous plexus were not observed. In view of the current conception of the visceral efferent mechanism, *two enteric neurons which sustain a synaptic relationship with each other can not be interpreted as terminal links in visceral efferent chains.* They were, therefore, interpreted as the components of local reflex arcs.

Whether enteric reflex arcs comprise intercalated neurons could not be determined in the histological preparations, because the synapse can be clearly seen only where the axon of a deeply stained neuron terminates on a neuron which is lightly stained. Consequently, the axon of the latter neuron can not be traced to its termination. However, whether a local reflex arc comprises two or three neurons is for the purposes of the present study a matter of only secondary importance. Synaptic relationships between two enteric neurons, as described above, seem to be sufficient to establish enteric reflex arcs as an anatomical fact.

As stated above, fibers may be traced from the submucous plexus into the muscularis mucosae, among the gastric and intestinal glands and into the gastric and intestinal epithelium. The fibers which terminate in the muscularis mucosae and among the glands must be primarily efferent. Those which terminate in the digestive epithelium are probably afferent. They probably represent dendrites of neurons, mainly in the submucous plexus, which constitute the afferent limbs of local reflex arcs. However, it need not be assumed that the afferent limbs of all enteric reflex arcs communicate with the digestive epithelium. Probably many of them arise in the muscle layers.

The anatomical relationships pointed out above suggest reflex arcs which

comprise *a*, only neurons in the same plexus, and *b*, reflex arcs which comprise a neuron in the submucous and another in the myenteric plexus. Such reflex arcs would constitute a mechanism through which stimuli arising in the lumen of the digestive tube or the musculature might result in glandular activity or motor responses involving all the muscle layers. Likewise, more localized reflexes carried out through the arcs which comprise only neurons in the submucous plexus might result in glandular activity or localized contractions of the muscularis mucosae. The existence of a local correlating mechanism is not incompatible with the anatomical findings and is positively indicated by the results of the present series of experiments and the data cited from the work of others.

In his recent experimental anatomical studies Johnson (1925) found no evidence of synapses in the enteric plexuses except the intercellular network of fine non-medullated fibers in the myenteric ganglia which disappears following section of the vagus nerves. He interpreted this finding and failure to observe synapses between local elements as indicating the absence of any synapses in the enteric plexuses except those of the preganglionic vagus fibers with enteric neurons. This is in complete accord with the current teaching, according to which the entire sympathetic system is purely efferent, and opposed to our conception of reflex arcs in the enteric plexuses.

As pointed out above, the axon of an enteric neuron which terminates by arborization around the cell body of another neuron in the same or a neighboring ganglion is quite independent of the intercellular network of fine fibers in the ganglia. The fact that this network drops out following section of the vagus nerves has no direct bearing on our findings. We are not unmindful of the fact that if local reflex arcs occur in the enteric plexuses, the structural pattern of these plexuses must differ somewhat from that of other parts of the autonomic nervous system. Neither have we disregarded the various attempts which have been made to account for known reflex activities of the digestive tube on the basis of some structural mechanism other than a system which includes local reflex arcs. Yet, in view of the positive observation of synaptic relationships between enteric neurons and the data cited in the present communication, we can not avoid the conclusion that a local reflex mechanism plays an important rôle in the functional activities of the digestive tube.

b. Rhythmic contractions in the stomach and intestine in relation to the enteric nervous system. The persistence of segmenting contractions in the small intestine, peristalsis in the stomach and anti-peristalsis in the large intestine, following paralysis of the nervous mechanism by means of nicotin indicates that these activities may be carried out by the gastro-intestinal musculature in the absence of nervous influences.

The methods employed in this phase of the present investigation have

certain definite advantages over those employed in earlier investigations. The criteria used as evidence of denervation of the muscle were more certain. Both the stomach and intestine were studied in situ. In the excised pieces of intestine used the normal relationships of the muscle layers to each other were preserved and excessive trauma such as necessarily results in any attempt to separate the muscle layers was avoided. The most peripheral known elements of the nervous mechanism were repeatedly subjected to definite functional tests designed to indicate the degree of irritability remaining until loss of nervous function was complete. Earlier investigators, e.g., Bayliss and Starling (1899) relied largely on abolition of the myenteric reflex as an indication of paralysis of the nervous mechanism. As stated above, the myenteric reflex is abolished by doses of nicotin much smaller than are required to abolish certain other functions of the enteric nervous mechanism. The doses of nicotin employed in the present series of experiments probably abolished the functions of the neuromuscular junctions and thus rendered further stimulation of either these structures or the terminal nerve-fibers ineffective. These terminal elements of the nervous mechanism might remain intact even in preparations of gastric or intestinal muscle separated from the myenteric plexus by dissection and still play a part in the activity of the muscle in the presence of appropriate stimuli.

We know of no previous investigation in which rhythmic contractions stimulating peristalsis were observed in the completely denervated stomach. Cannon (1909) observed rhythmic contractions in the stomach following "large" doses of nicotin. However, he does not state the dosage employed. Neither do we know of any previous records of rhythmic contractions in the small intestine in situ following procedures which were certainly adequate to completely paralyze the enteric nervous mechanism. The methods of denervation previously employed which most nearly approach in effectiveness the method employed by us, involve excision and severe trauma of the tissue as well as separation of the muscle from its normal blood supply. Furthermore, the abnormal mechanical conditions associated with graphic registration are probably less disturbing in experiments with the stomach and intestine in situ than with excised preparations by reason of the fact that in the former the muscle contracts against a body which bears somewhat the same relation to the wall of the viscus as does its normal content.

We recognize the objections which may legitimately be raised against the use of drugs in the study of physiological phenomena. However, the drug employed in the present series of experiments to bring about paralysis of the nervous mechanism could not vitiate the results unless it should itself induce the muscular activity observed following its use. We know of no experimental data which indicate that nicotin directly excites

rhythmic contractions in resting smooth muscle cells. On the contrary, some of the data set forth in this paper indicate that, in the dosage used, it has an exactly opposite effect, viz., a depressant action on the rhythmic contractions of smooth muscle. As stated above, the rhythmic contractions of excised pieces of intestine were retarded in solutions containing 0.2 per cent, and abolished in solutions containing 0.5 per cent of nicotin.

In so far as the results of our experiments indicate that the gastro-intestinal musculature possesses the inherent capacity to contract rhythmically, they corroborate the findings of those investigators who regard the rhythmic contractions of the stomach and intestine as myogenic. On the other hand, they neither indicate that the rhythmic contractions of the stomach and intestine are normally carried out without nervous control nor that the gastro-intestinal musculature could adequately perform even its simpler motor functions in the absence of nervous influences. The rhythmic contractions which persist in these organs after the nervous system is paralyzed differ widely from those occurring in the unpoisoned organs. Whereas records of even the simplest forms of rhythmic activity in an unpoisoned segment of the intestine, in which it may be assumed that the nervous elements remain functional, are characterized by frequent changes in tonus and amplitude which are so varied and complex as practically to defy analysis, all such irregularities in the record disappear following denervation with nicotin. The activity which persists consists of mechanically regular contractions and relaxations. While the record of the former can not be regarded as representing strictly normal functional activity, nevertheless, the difference between the extremely variable activity seen in the normal viscus and the mechanical regularity displayed by the denervated preparation probably represents the activity of the peripheral nervous mechanism. The apparently aimless changes seen in the activity of the unpoisoned tissues under experimental conditions probably represent the functional activity of a nervous mechanism qualified to produce similar changes in an orderly and purposeful manner when subjected to the stimuli of its natural environment.

Both in our experiments with excised pieces of intestine and with the stomach and intestine in situ, as the dosage of nicotin was progressively increased, the amplitude of the rhythmic contractions increased progressively until the dosage of nicotin became relatively high, and then gradually decreased. If it may be assumed that the influence of the smaller doses of nicotin is exerted mainly on the nervous mechanism, this fact suggests a functional relationship of the enteric nervous system to the amplitude of the rhythmic contractions. The fact that all contractions cease in very high concentrations of nicotin suggests that the reduction in the amplitude of the contractions during the later part of our experiments was due to a depression affecting the muscle directly. The cause of the

increase in amplitude which preceded this depression is less obvious. It may be due to primary stimulation of the muscle preceding the paralysis. This would be in keeping with the fact that other structures are primarily stimulated and secondarily depressed by nicotin. On the other hand, the gastro-intestinal musculature may normally be subject to inhibitory influences exerted by the enteric nervous mechanism. Such inhibitory influences would be removed as soon as the nervous mechanism became materially depressed by nicotin. The weight of concrete evidence favors the latter possibility. Preparations of intestinal muscle when first set up are usually quiescent and begin to contract only after a variable interval. The interval of quiescence may be increased by manipulation and trauma. On the other hand, it may be terminated promptly by the administration of sufficient nicotin to materially depress the nervous mechanism. Possibly both stimulation of the muscle and removal of nervous inhibition play a part. However, the rate of the contractions is not increased in proportion to the amplitude. Nervous stimulation has no constant effect on the rate of contraction. On the other hand, the depressant effect of nicotin in high concentration is apparent both on the amplitude and the rate of the contractions. These facts suggest that the removal of inhibition may be more important quantitatively in increasing the amplitude than direct stimulation of the muscle. They also suggest that the inhibition which is usually considered to be responsible for the quiescence of the gastro-intestinal musculature following operative procedures or manipulation is due not alone to reflexes involving the central nervous system, but, as Bayliss and Starling believe, may be brought about also by inhibitory influences exerted by the enteric nervous system.

The strong contrast between the relative constancy of the rate of the rhythmic contractions and the variability of tonus and amplitude under experimental conditions, including nervous stimulation by means of the electric current or by the action of drugs, suggests that the rate may be independent of the nervous influences which bring about changes in amplitude and tonus. The rate of the rhythmic contractions probably depends on properties which are inherent in the gastro-intestinal musculature, and is not subject to nervous control in the same degree as tonus and amplitude. The decrease in rate which we observed following the administration of nicotin in massive doses was probably due to the depressant action of the drug on the muscle. Alvarez (1918) states that, in his experiments, the rate of the contractions was increased somewhat by the action of nicotin. However, he does not state the dosage employed and the changes in rate which he observed were slight.

The persistence of the rhythmic contractions in the stomach and intestine, after intestinal peristalsis and certain other coördinated reflexes have been abolished by the use of nicotin or other drugs has seemed to

some investigators to exclude the functioning, in these activities, of a nervous mechanism involving synapses. If the assumption were correct that the abolition of the myenteric reflex and the interference with conduction over the vagus path which results from such doses of nicotin as were used by Bayliss and Starling (1899) indicate paralysis of the entire nervous mechanism, any other view would indeed be untenable. However, as pointed out above, we found that one of the effects of nicotin, namely, the loss of vagus function, is a transient result which occurs only with moderate doses and disappears when the dose is materially increased. Apparently the synapses of the vagus fibers with myenteric neurons are not paralyzed by the smaller doses of nicotin, such as were used by Bayliss and Starling, but their function is interfered with in some other way, possibly by inhibition. Moreover, the dose of nicotin required to finally paralyze the vagus synapses (2 or 3 grams per kilo) is as great as the dose required to paralyze the neuromuscular junctions of the post-ganglionic sympathetics. Consequently, there must be synapses in the nervous mechanism which are as resistant to nicotin paralysis as the neuromuscular junctions themselves. Therefore, it is possible to explain the continuance of function in the enteric plexuses even after the administration of nicotin in small doses without assuming protoplasmic continuity of the nervous elements and the theory that the enteric nervous mechanism functions essentially as a synaptic reflex system, even in its control of the rhythmic contractions, is rendered tenable. We know of no experimental findings which, in the light of the results of the present investigation, are incompatible with this theory. On the other hand, we believe that all data set forth may be more satisfactorily interpreted on the basis of this theory than on the basis of any other conception of the structure of the enteric plexuses which has hitherto been advanced.

SUMMARY AND CONCLUSIONS

The motor activities of the stomach and intestine may be classified as follows: 1, movements which are obviously reflex, including peristalsis both in the small and large intestine and many other coördinated contractions; 2, rhythmic contractions, including gastric peristalsis, segmenting contractions in the small intestine, and anti-peristalsis and other rhythmic contractions in the large intestine.

Gastro-intestinal movements of the former class are abolished by paralysis of the enteric nervous system. They can be explained most satisfactorily on the theory that the enteric nervous system comprises local reflex arcs. This theory is further substantiated by the anatomical studies of one of the present writers (Kuntz, 1922) in which synaptic relationships, such as would be required in a system comprising local reflex arcs, are described in the enteric plexuses.

The rhythmic gastric and intestinal movements persist following the administration of nicotin in doses sufficient to paralyze the most peripheral elements of the enteric nervous mechanism. Therefore, the capacity to contract rhythmically seems to be an inherent property of the gastro-intestinal musculature.

The tonus of the muscle and the amplitude of the contractions undergo frequent changes, under experimental conditions, while the enteric nervous mechanism remains functional. Such changes do not occur following complete paralysis of the enteric nervous system. The rate of the rhythmic contractions is not materially altered by conditions affecting the nervous mechanism, but is altered by conditons affecting the muscle.

The experimental data seem to justify the following conclusions. 1. While the capacity to contract rhythmically is probably inherent in the gastro-intestinal musculature motility of this type would be functionally inadequate in the absence of nervous control. 2. The enteric nervous mechanism brings about variations in the tonus of the musculature and the amplitude and force of the contractions, and initiates and coördinates peristaltic and other reflex movements in response to local stimuli. These activities are, under normal conditions, subject to some degree of control by the central nervous system through the extrinsic nerves.

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ON THE NATURE OF URINARY GLUCOSE[1]

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Much interest has followed the findings of Hewitt and Pryde (1) that glucose solutions change in optical value when allowed to remain for brief periods in contact with intestinal mucosa. These two workers found that there was a decrease in specific rotation of the glucose during its contact with living mucosa, and a corresponding increase in rotatory power of the sugar after removal from the segment. This last change in rotation continued until the amount of glucose calculated from the copper reduction value and optical value, for the well-known equilibrated mixture of alpha and beta forms of glucose, coincided. The decrease in optical value was said to be due to the formation of the very reactive ethylene oxide form of glucose by the influence of the living intestine; the change of rotation in an upward direction was ascribed to a reversion to alpha and beta glucose. Stiven and Reid (2) have failed to confirm the findings of Hewitt and Pryde. More recently Hume and Denis (3) have reported negative results. They observed higher copper reduction than polariscopic values for glucose content.

Winter and Smith (4) have attempted to demonstrate the presence in normal human blood of the reactive form of glucose. These investigators observed that concentrated extracts of residues from evaporation of protein-free blood filtrates showed a progressive increase in rotation until approximation of copper reduction value was obtained by polariscopic measurement. Similar solutions from diabetic blood showed either no change in rotation or a change in downward direction. Forrest, Winter and Smith (5) later reported that insulin was able to change the nature of the curve for diabetic blood to correspond with that for normal blood. The above workers implied that normal blood contained the ethylene oxide form of glucose, which the English workers have termed "gamma," while diabetic blood contained only the alpha and beta forms, which insulin was able to alter in configuration and properties. The above conception was criticised by Hewitt (6) and reaffirmed by Winter and Smith (7). Repeti-

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tion of the procedures of Winter and Smith by Eadie (8), Denis and Hume (9) and Visscher (10) have failed in confirmation of the conception.

Such varied results found by different workers may have been due to:

1. Changes in pH of filtrates, suggested by Visscher.

2. Too small an amount of glucose present to give detectable differences in polariscopic readings.

3. Presence of other optically active substances besides glucose in concentrated filtrates.

TABLE 1

Urine samples from phlorhizinized dogs. No insulin

EXPERIMENT NUMBER	POLARISCOPE READING	TIME AFTER CATHETERIZATION	GLUCOSE IN 100 CC. URINE BY		REMARKS
			Polariscope	Copper reduction	
			grams	*grams*	
1	2.30	42 mins.	1.52	1.32	
	2.30	5½ hrs.	1.52		
	2.30	27 hrs.	1.52		
2	10.00	42 mins.	6.61	6.19	Fed before catheterization
	10.00	25 hrs.	6.61	6.24	
	9.90	40 hrs.	6.54		
3	3.03	70 mins.	2.00	1.92	
	2.88	66 hrs.	1.90		
4	3.25	38 mins.	2.15	2.04	
	3.30	5¾ hrs.	2.18		
5	6.50	52 mins.	4.29	4.12	Fed before catheterization
	6.70	25 hrs.	4.43		
6	3.24	55 mins.	2.14	1.96	
	3.07	67 hrs.	2.02		

4. Reversion of any ethylene oxide form of glucose to more stable alpha and beta mixture by evaporation to dryness.

5. Contamination of filtrates by molds and bacteria.

It has occurred to us that the relatively large amount of glucose in the urine of phlorhizinized animals might exclude some of the above hazards, as well as shorten by several hours the time of manipulation of a glucose solution before the first polariscopic reading. This report compares the optical values of such urine samples with their copper reduction values, both with and without the influence of insulin.

EXPERIMENTAL PROCEDURE. *Treatment of animals.* Female dogs were operated upon to facilitate catheterization of urine. After recovery the

animals were starved for three days. They received next subcutaneous injections of 1 gram of Merck's phlorhizin in 5 cc. olive oil. Wherever indicated in the accompanying tables the animals were fed with a mixture of 100 grams of casein, 50 grams of glucose and 500 cc. of milk. This type of food was found to serve the double purpose of maintaining an acid urine

TABLE 2

Urine samples from phlorhizinized dogs, using insulin

EXPERIMENT NUMBER	POLARISCOPE READING	TIME AFTER CATHETERIZATION	GLUCOSE IN 100 CC. URINE BY		REMARKS
			Polariscope	Copper reduction	
			grams	*grams*	
1	9.08	63 mins.	5.99	5.61	Fed before injection of 20 units insulin
	8.95	20 hrs.	5.91		
2	12.57	40 mins.	8.30	8.00	Followed experiment 1
	12.50	19 hrs.	8.26		
3	13.60	52 mins.	8.98	8.64	Fed before injection of 40 units insulin
	13.26	45 hrs.	8.76		
4	12.42	70 mins.	8.20	7.73	Continued experiment 3
	12.45	42 hrs.	8.22		
5	3.47	70 mins.	2.29	2.14	Continued experiment 4
	3.37	41 hrs.	2.23		
6	4.08	41 mins.	2.73	2.63	Fed before injection of 20 units insulin
	4.11	21 hrs.	2.72		
7	3.77	30 mins.	2.49	2.30	Continued experiment 6
	3.69	19 hrs.	2.44		
8	6.62	15 mins.	4.37	4.20	Fed before injection of 40 units insulin
	6.74	46 hrs.	4.45		
9	2.91	60 mins.	1.92	1.73	Continued experiment 8
	2.67	44 hrs.	1.76		
10	2.55	60 mins.	1.68	1.66	Continued experiment 9
	2.61	18 hrs.	1.72		

and also of fortifying the animal against hypoglycemic symptoms from insulin injection. Iletin, of Eli Lilly & Co., was injected subcutaneously only as indicated in the table 2. Before an experimental period the bladder was emptied and the urine discarded.

Treatment of samples. After an experimental period of one to two hours the specimen for examination was drawn and clarified immediately. Many

clarifying reagents were employed before one adaptable to the methods used was tried. Finally Lloyd's reagent was found to supply the demands for production of very clear and permanent filtrate. It was used in the manner recommended by Folin (11) for removing substances from urine, examining same for small amounts of glucose. The sample was diluted with an equal volume of 0.1N sulphuric acid, and shaken for several minutes with 5 grams of Lloyd's reagent for every 100 cc. of liquid. The filtrates from such treatment were so nearly colorless and permanently clear that they could be easily examined in a 4-dm. tube in polariscope. The filtrates were preserved with toluol and it was found more expedient to use two decimeter polariscope tubes. Solutions were examined immediately after filtration, in 30 to 70 minutes, with use of a Bates saccharimeter as designed by the Bureau of Standards. We are indebted to Professor McGuigan, of the University of Illinois, for use of a Bates saccharimeter. Illumination was supplied by a 100 c.p. frosted electric bulb. Later polariscopic readings were made upon portions of the same solution, or on the same solution. The temperature of solutions examined in this manner was 20 to 25°C. Shortly after the first polariscopic reading a portion of the same solution was examined for copper reduction value by the method of Schaffer and Hartman (12). All values obtained were made in duplicate and the tables present averages of satisfactory agreements. Only acid urines were examined, although hydroxybutyric was never detectable by qualitative methods. The foregoing tables summarize the results. All optical values are positive and given in degrees Ventzke, which must be multiplied by the factor of 0.3468 to obtain angular rotation.

Discussion of results. If the kidneys do not function in changing the configuration of glucose from that existent in blood, and if injection of phlorhizin does not alter the properties of blood sugar, one might be warranted in concluding from results of table 1 that such a type of reactive mutarotatory "body" glucose is not present in the blood. The results offer closer agreement between polariscopic values, which remain constant for hours, and copper reduction values than has hitherto been obtainable. Until the ethylene oxide form of glucose is administered to normal and diabetic animals the question of kidneys altering properties of blood sugar must remain an open one.

Assuming that phlorhizin so impairs normal mechanisms that "body" glucose can no longer be produced, it is interesting to note that insulin does not bring about any change in the nature of blood sugar, if the nature of urinary glucose mirrors that of the blood. Recent publications by Nash and Benedict (13), (14), Ringer (15) and Colwell (16) are of interest in this connection. Nash and Benedict assert that the phlorhizinized animal is unable to utilize insulin to a measurable degree for glucose retention or consumption, and report insulin present in pancreatic tissue of such animals. Ringer and Colwell, however, were able to discern glucose

utilization by the phlorhizinized dog and the former suggests that such an animal may be used to assay insulin preparations.

CONCLUSIONS

1. Polariscopic and copper reduction values show close agreement over long periods of time, when used to measure quantity of glucose in the urine of the phlorhizinized dog.

2. As measured by polariscopic and copper reduction methods the urine of phlorhizinized dogs, not receiving insulin, and receiving insulin injections, contains only the alpha and beta types of glucose in equilibrium. Such methods show no ethylene oxide form of glucose in urine, or blood.

Since the completion of the above experiments, Lundsgaard and Holboell (17), (18) have found that the incubation of glucose with insulin and muscle juice has resulted in a decrease of the specific rotation of the glucose from the value of $+52.5°$ for the alpha and beta mixture. In view of their findings and the results reported above, one would be inclined to the opinion that phlorhizin either poisons the reaction of glucose alteration by insulin and muscle or that the kidneys are able to excrete the alpha and beta forms of glucose, while retaining the form of different rotation.

Since we completed the work described above Tallerman has reported close agreement of copper reduction and polariscopic measurements on urine samples obtained from man after injection of 0.005 gm. of phlorhizin (19). This worker has not described a method for clarifying urine specimens for polariscopic examination; neither did he administer insulin to the phlorhizinied subject for the purpose described above.

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BLOOD CLOTTING STUDIES IN HEMOPHILIA

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Vines (1920) accidentally discovered that hemophiliacs were temporarily rendered normal by inducing a local protein reaction in the skin. He was preparing to make a second injection of horse serum into a hemophiliac boy to stop bleeding, and took the precaution of first making an intradermal injection to test for previous sensitization. The skin test was strongly positive. The bleeding promptly ceased with the protein reaction, the clotting time shortened, and remained almost normal for about a month. This treatment he tried on three other hemophiliacs with greater or less success. He could offer no lucid explanation of it, and made no studies of the clotting factors of the blood. Howell has pointed out that the principal abnormality of hemophiliac blood lies in its great deficiency in prothrombin. We, therefore, desired to see whether the treatment used by Vines was successful because of a prothrombin generation in the blood.

We have reported elsewhere (Mills et al., 1923) the effects of tissue fibrinogen on the clotting time of the blood of hemophiliacs. The reduction in clotting time is roughly the same here as with patients possessing normal blood,—that is, the reduction may be from 15 minutes down to 5 minutes, instead of from 3 down to 1 minute. Although the hemorrhagic tendency is greatly diminished and many hemorrhages well controlled, the patients are still left with a clotting time well above normal. Also the tissue fibrinogen effect is usually of only a few hours' duration, in no sense producing a cure of the condition. We therefore wished to examine further into the source of trouble in hemophilia, and particularly to secure an explanation for Vines' results. Hope of more permanent betterment for these patients probably lies along that line of research.

Work on hemophilia in the past has centered mainly on three points: 1, a deficiency of thombokinase in the blood (Sahli, 1910) (Morawitz and Lossen, 1908); 2, the presence of an inhibitor substance preventing thrombin production (Weil, 1905), (Feissly, 1924); and 3, an inadequacy of prothrombin either in amount or ability to convert into thrombin. Howell (1914) holds that the trouble lies in an inadequate supply of prothrombin, the inhibitory substances of the blood not being increased. Hurwitz

and Lucas (1916) support the findings of Howell, while Addis (1911) claims to have demonstrated a normal amount of prothrombin to be present, but its conversion to thrombin to be only about one-third the normal rate.

The results of our work presented below confirm Howell and Addis in that no excess inhibitory substance is to be found, but lead to no clear-cut conclusions regarding the prothrombin question. In the fresh serum from a hemophiliac clot a large amount of active thrombin is found, but the addition of active cephalin to this serum at no time causes a production of additional thrombin. Likewise cephalin fails to accelerate the clotting of the whole blood. This entire failure of cephalin effect seems to be the most constant and characteristic finding with such bloods. The interesting feature of this work is that the induction of a protein skin reaction causes the production of prothrombin which reacts normally toward cephalin. Tissue fibrinogen we found to cause as great a reduction in the clotting time of hemophiliacs' blood as it did in normal blood, and this effect was not increased after a normally reacting prothrombin had been developed in the blood of the hemophiliac. This supported our views regarding the existance of two independent clotting mechanisms, that from tissue fibrinogen union with blood fibrinogen, and that from thrombin action.

The bloods of four typical hemophiliac children were available for our study. The personal history of each of these children, together with the blood findings, left no room for doubt as to their being true hemophiliacs although no positive family history was obtainable in two instances.

F. B., K 1178 (white male, 14 months old) had been in the Cincinnati General Hospital for about 2 weeks recovering from a fairly severe hemorrhage which had resulted from a torn upper labial frenum. His red cell count had risen to over three million and he was ready to be discharged at the time the blood sample was taken. Fifteen cubic centimeters of blood were drawn from the arm vein, citrated to 0.5 per cent and centrifuged to obtain clear plasma. This plasma was used in the tests below (B.P.). The cephalin used was a 0.5 per cent emulsion of cephalin purified from sheep brain. The horse plasma (H.P.) used was citrated to the same concentration as the human plasma.

0.5 cc. B.P. + 00 cephalin + 0.1 cc. 1 per cent $CaCl_2$, solid clot in 34 minutes.
0.5 cc. B.P. + 0.2 cc. cephalin + 0.1 cc. 1 per cent $CaCl_2$, no clot in 1½ hours.
0.5 cc. B.P. + 0.2 cc. tissue fibrinogen + 0.1 cc. $CaCl_2$, solid clot in 3 minutes.

These results may be compared to similar results on citrated horse plasma (H.P.).

0.5 cc. H.P. + 00 cephalin + 0.1 cc. 1 per cent $CaCl_2$, clot in 15 minutes.
0.5 cc. H.P. + 0.2 cc. cephalin + 0.1 cc. 1 per cent $CaCl_2$, clot in 9 minutes.

0.5 cc. H.P. + 0.2 cc. tissue fibrinogen + 0.1 cc. $CaCl_2$, clot in 1½ minutes.
0.5 cc. H.P. + 0.5 cc. B.P. + 0.2 cc. 1 per cent $CaCl_2$, clot in 3 minutes.

The tissue fibrinogen effect is about the same on the hemophiliac plasma (reducing the clotting time from 34 minutes down to 3 minutes) as on the horse plasma (reducing from 12 down to 1⅓ minutes). The horse plasma used here had stood in the ice box for several days, which accounts for its prolonged clotting time. The effect of the cephalin on the two plasmas was entirely different. On the horse plasma it exerted its usual moderate effect, while on the hemophiliac plasma it caused a marked inhibition of clotting.

The absence of any inhibitor of clotting in F. B.'s blood is demonstrated in the last test, where a mixture of equal parts of the two plasmas is seen to clot much better than either separately.

Serum was next obtained from F. B.'s plasma and examined for thrombin factors.

4 cc. of B.P. + 0.2 cc. tissue fibrinogen + 0.8 cc. 1 per cent $CaCl_2$, clotted—serum saved.
0.5 cc. H.P. + 0.25 cc. of this serum (7 minutes old), clot in 30 seconds.
0.5 cc. H.P. + (0.25 cc. serum + (7 minutes old) + 1 drop cephalin) clot in 30 seconds.
0.5 cc. H.P. + 0.25 cc. this serum (23 minutes old), weak clot in 25 minutes.
0.5 cc. H.P. + [0.25 cc. of this serum (23 minutes old) +1 drop cephalin], weak clot in 25 minutes.

There is thus seen to be a moderate amount of thrombin present in this serum, disappearing much more rapidly, however, than does thrombin in normal serum. The striking fact seen here is the absolute lack of cephalin effect. Not the slightest increase in thrombin content of the serum was noticed after cephalin addition. In normal serum we show elsewhere (Mills, 1926) that a prothrombin regeneration accompanies the thrombin disappearance from serum, so that the cephalin effect becomes even slightly more marked after the serum has aged for a while. In neither the fresh, nor somewhat older, hemophiliac serum was any cephalin effect detectable.

In another article in this number of the JOURNAL (Mills), we show that serum added to recalcified citrate plasma clots this plasma in about half the time required for it to clot the same plasma without recalcification. We interpreted this as meaning that in the recalcified plasma the prothrombin of the plasma was converted to thrombin, so that the fibrin was produced by both the added thrombin of the serum and the new thrombin produced in the recalcified plasma. This experiment was tried on F. B.'s plasma, with the following result.

1 cc. B.P. + 0.1 cc. tissue fibrinogen + 0.2 cc. 1 per cent $CaCl_2$ cc.—serum saved.

0.5 cc. B.P. + 0.25 cc. above serum, clot in 25 seconds.
0.5 cc. B.P. + 0.1 cc. 1 per cent $CaCl_2$ + 0.25 cc. above serum, clot in 30 seconds.

The recalcified plasma clotted certainly no more rapidly than did the uncalcified, so we must conclude that no thrombin production occurred to take part in the clotting. We are as yet at a loss to account for this apparent lack of thrombin production here, when we see a considerable amount of it produced in the same plasma when clotted by tissue fibrinogen and calcium.

E. H., K 959, 13 year old white boy, was admitted to the Cincinnati General Hospital, February 7, 1925, for the extraction of several deciduous teeth. He gave a history of severe bleeding following slight injuries at various times during his life. No positive family history was obtained. His clotting time on admission was about 20 minutes (tested by the method of Mills and Peterson, 1923). The blood cells and platelets were normal in number. He received tissue fibrinogen orally before each meal for a week, at the end of which time his clotting time was about 10 minutes. A few of the smallest decidual teeth were lifted off the gums with very slight bleeding at the time and for 12 hours afterward. A slow continuous ooze for the following 2 days developed the need of transfusion. Two transfusions of citrated blood (240 cc. and 400 cc.) from different donors produced no effect whatever on the bleeding, but a third of 350 cc. from a third donor caused a prompt cessation of the hemorrhage. The boy regained his strength rapidly and was up and about in 3 weeks. There was some slight bleeding around two badly decayed teeth.

It was decided at this time to try the treatment of Vines on the boy. A first sample of blood was drawn and citrated for tests as indicated below. He was then given an intradermal injection of 2 drops of normal horse serum. No reaction occurring, he was given 4 cc. intramuscularly on the same day (April 4, 1925). One week later the intradermal injection was repeated, producing a fairly marked local reaction, with a raised wheal about 2 cm. across lasting several hours. No further bleeding around the teeth was to be seen. Still another intradermal injection was given a week later and a fair reaction obtained. The second sample of blood was drawn just following this last injection. The results of tests on both these blood samples follow, sample 1 coming first.

0.5 cc. E. H.'s P. + 00 + 0.1 cc. 1 per cent $CaCl_2$, clot in 90 minutes.
0.5 cc. E. H.'s P. + 0.05 cc. brain extract + 0.1 cc. 1 per cent $CaCl_2$, clot in $1\frac{1}{4}$ minutes.
0.5 cc. E. H.'s P. + 0.1 cc. pure cephalin + 0.1 cc. 1 per cent $CaCl_2$, clot in 43 minutes.

The tissue extract is seen to reduce the clotting time almost to the same point as it would that of normal blood, while the cephalin effect is very

weak. There is no cephalin inhibition however. Addition of E. H.'s plasma to normal horse plasma somewhat slows its clotting rate, as is now shown.

0.5 cc. horse plasma + 00 + 0.1 cc. 1 per cent $CaCl_2$, clot in 15 minutes.
0.5 cc. horse plasma + 0.5 cc. E. H.'s P. +0.1 cc. 1 per cent $CaCl_2$, clot in 21 minutes.

The thrombin content of the serum was examined in the same fashion as with F. B.'s serum.

3 cc. E. H.'s P. + 1 drop of brain extract + 0.6 cc. 1 per cent $CaCl_2$, clotted and serum saved.

0.5 cc. Horse P. + 0.25 cc. of this serum (20 seconds old), clot in 8 seconds.
0.5 cc. H.P. + 0.25 cc. of this serum (3 minutes old), clot in 35 seconds.
0.5 cc. H.P. + 0.25 cc. of this serum (5 minutes old), clot in 1 minute 5 seconds.
0.5 cc. H.P. + (0.25 cc. of this serum (5 minutes old) + 0.1 cc. cephalin) clot in 2 minutes 20 seconds.
0.5 cc. H.P. + 0.25 cc. of this serum (12 minutes old), clot in 2 minutes 20 seconds.
0.5 cc. H.P. + 025 cc. of this serum (45 minutes old), no clot.
0.5 cc. H.P. + [0.25 cc. of this serum (45 minutes old) +0.1 cc. cephalin], no clot.

The amount of thrombin in the fresh serum from E. H.'s plasma seems to be considerable, (clotting citrated horse plasma in 8 seconds) but it rapidly disappears and at no time in the course of its disappearance can any additional thrombin be produced by addition of fresh cephalin to the serum. This lack of cephalin activation seems to be quite a typical finding in hemophiliac serum.

Tests on the second sample of E. H.'s blood taken after the protein sensitization and reaction indicated that a definite change in the blood had taken place.

0.5 cc. E. H.'s P. + 00 + 0.1 cc. 1 per cent $CaCl_2$, clot in 48 minutes.
0.5 cc. E. H.'s P. + 0.1 cc. brain extract + 0.1 cc. 1 per cent $CaCl_2$, clot in 1 minute 25 seconds.
0.5 cc. E. H.'s P. + 0.1 cc. cephalin + 0.1 cc. 1 per cent $CaCl_2$, clot in 14 minutes.

The cephalin effect is seen to be much greater than it was on the first sample of his blood, while the tissue extract effect remains about the same. Whereas the first sample inhibited the clotting of normal horse plasma, this second plasma definitely accelerated it (see below).

0.5 cc. horse plasma + 00 + 0.1 cc. $CaCl_2$, clot in 15 minutes.
0.5 cc. horse plasma + 0.5 cc. E. H.'s plasma + 0.1 cc. $CaCl_2$, clot in 7 minutes 30 seconds.

Thrombin tests on the serum were carried out as before:

3 cc. E. H.'s P. + 1 drop brain extract + 0.6 cc. $CaCl_2$, clotted and serum saved.

0.5 cc. Horse P. + 0.25 cc. of this serum (1 minute old), clot in 20 seconds.

0.5 cc. Horse P. + [0.25 cc. serum (2 minutes old) + 1 drop cephalin], clot in 12 seconds.

0.5 cc. Horse P. + [0.25 cc. serum (3 minutes old) +1 drop cephalin], clot in 15 seconds.

0.5 cc. Horse P. + [0.25 cc. serum (13 minutes old) +1 drop cephalin], clot in 35 seconds.

0.5 cc. Horse P. + 0.25 cc. serum (13 minutes old), clot in 6 minutes very weakly.

Here we obtained the first cephalin activation of hemophiliac serum. The effect was quite marked, serum 13 minutes old that clotted plasma only weakly after 6 minutes, was made to clot it solidly in 35 seconds. Another sample of this serum that gave only a trace of clot in 40 minutes was activated by cephalin to give solid clotting in 50 seconds. This serum is, of course, still far from normal in its prothrombin content, but the encouraging point is that there has been generated a prothrombin capable of cephalin activation.

This normal behavior of his prothrombin in the latter blood sample is brought out in a test like that carried out in F. B.'s plasma.

1 cc. E. H.'s P. + 0.25 cc. this serum (1 minute old), clotted in 31 seconds.

0.5 cc. E. H.'s P. + 0.1 cc. $CaCl_2$ + 0.25 cc. this serum (1 minute old), clotted in 16 seconds.

The reaction here was perfectly normal. That is, in the recalcified plasma the clotting was about twice as rapid as in the uncalcified. As explained elsewhere, this indicates a rapid conversion of the prothrombin of the plasma to thrombin, the final clotting representing the action of this new thrombin together with the thrombin in the serum added. Normal human and horse plasmas give results quite similar to the above, so we may consider E. H.'s plasma as now containing normal prothrombin. It is unfortunate that his first blood sample was not similarly tested to check these findings. F. B.'s plasma, however, gave entirely negative results for thrombin production under similar circumstances, and E. H.'s first sample seemed in most respects quite similar to F. B.'s. We may infer, then, that this thrombin production under the above conditions would not have taken place in E. H.'s first sample as it did in his second.

In all respects his blood is now similar to normal blood except for a slightly low prothrombin content. The prothrombin present behaves quite normally. His clotting time is now $4\frac{1}{4}$ minutes (normal is $2\frac{1}{2}$ to 3 minutes by the method used). He will be followed as long as possible to determine the duration of this effect on the prothrombin and on his bleeding tendency.

D. H., K 8852, age 19 years, hemophiliac, was admitted to the Cincinnati General Hospital November 20, 1925, bleeding from three sockets following teeth extraction the preceding day. He gave a history

of previous attacks of protracted bleeding and had a maternal uncle likewise afflicted. The bleeding was slow but constant. His clotting time was 5 minutes, bleeding time 4 minutes, platelets 350,000. He was given an intracutaneous injection of 3 minims of horse serum on the day of admission, and later, no reaction developing, 4 cc. of the same serum subcutaneously to produce sensitization. Local measures to stop the bleeding were without avail, and the need for transfusion developed before he had become sensitized to the serum. We were anxious to try the effect of the protein reaction on the bleeding, but had to resort to transfusion before this was possible. On November 22nd, he received 400 cc. of blood by the citrate method from a brother, with practically no effect on the bleeding. This was repeated 3 days later, using a second brother, but again without effect. On November 28th, one week after the subcutaneous serum injection, he was given another intradermal injection of 4 minims. There was no reaction whatever, indicating an entire lack of sensitization up to that time. About 5 hours later in the same day he received 800 cc. of blood from his brother-in-law, who was known to be sensitive to horse serum. This transfusion had no effect on the bleeding while it was being given, such as usually occurs if any effect is to take place. However, about 30 minutes after the transfusion was finished, it was noticed that a marked local reaction was beginning at the site of the serum injection 6 hours earlier. As this reaction developed, it was noted that the bleeding quite suddenly stopped and at no time recurred. The local protein reaction lasted about 3 hours, disappeared, and reapeared for short intervals 3 times in the succeeding week. No other symptoms of anaphylaxis or systemic protein reaction were present at any time. Following this reaction the patient made a good recovery and was discharged.

His clotting time on admission (method of Mills and Peterson) was 5 minutes. A sample of blood drawn at this time and citrated to 0.5 per cent was used for the following tests:

0.5 cc. of this plasma + 0.1 cc. 0.9 per cent NaCl + 0.1 cc. 1 per cent $CaCl_2$—clotted in 70 minutes.

0.5 cc. of this plasma + 0.1 cc. 0.5 per cent cephalin + 0.1 cc. 1 per cent $CaCl_2$—clotted in 65 minutes.

0.5 cc. of this plasma + 0.1 cc. 1.5 per cent tissue fibrinogen + 0.1 cc. 1 per cent $CaCl_2$—clotted in 55 seconds.

One-tenth cubic centimeter D. H.'s serum, 2 minutes old, did not clot 0.5 cc. horse plasma within 1 hour. A like sample of his serum, with cephalin added, did no better. The addition of 0.1 cc. 5 per cent Witte peptone to 0.1 cc. this serum and permitting to stand 5 minutes, gave a mixture which clotted the horse plasma in 1 minute and 30 seconds. The further addition of cephalin to such a serum-peptone mixture brought

on clotting of the plasma in 50 seconds. Here is, indeed, a rather startling effect of peptone in hemophiliac serum. The principal trouble seems to lie in the inability of cephalin to activate prothrombin to thrombin, and in the rapid and complete disappearance of thrombin from the serum. We see peptone to cause thrombin to reappear in a serum previously inactive, and we further see that this peptone-serum mixture forms still more thrombin when cephalin is added.

On December 12th, D. H.'s clotting time was 10 minutes. He was given 4 minims of horse serum intradermally. A good local reaction followed. One hour and 20 minutes later his clotting time was 2 minutes 30 seconds, entirely normal.

The local protein reaction was also tried on E. M., K 7288, another hemophiliac admitted to the hospital September 21, 1925. He had had horse serum injected some months previously, so we merely gave an intradermal injection of 4 minims. His clotting time September 25th, was 5 minutes. Serum injection was given and the clotting time 45 minutes later was 3 minutes 30 seconds. Clotting time October 4th, was 4 minutes. Serum injection was repeated. Clotting time 1 hour later was 2 minutes 40 seconds. He has not been seen since. In this case we again see the corrective influence of the local protein reaction on the hemophiliac condition.

Discussion. The work here recorded bears further proof of the presence of two clotting mechanisms as described by Mills and Mathews (1924). Here in hemophiliac blood, where there is such a paucity of prothrombin, and cephalin alone is quite ineffective, the tissue fibrinogen clotting goes on almost normally. After the protein sensitization and skin reaction in E. H. cephalin is seen to clot the recalcified plasma in 14 minutes as against 43 minutes in the first plasma sample; the tissue fibrinogen effect, however, is not at all quickened. The same is true with D. H. These two points indicate to us an independence of the two clotting mechanisms.

As to the significance of our results concerning thrombin and prothrombin in the hemophiliac blood, we are left quite uncertain. Immediately after this plasma has been clotted by tissue fibrinogen there is found in the serum quite a rich supply of active thrombin. It disappears from the serum very rapidly, however, and as it vanishes we find no corresponding regeneration of prothrombin such as is seen in normal serum. This can scarcely be due to a high antithrombin content of the serum, since when added to fresh normal horse serum, it does not accelerate the rate of thrombin disappearance. Howell also found no actual increase in the amount of antithrombin present.

Cephalin is practically without effect on the clotting of hemophiliac blood, and is entirely ineffective in causing a thrombin production in the

serum. We can assume, then, that, if prothrombin be present, it has not the character of normal prothrombin, for the latter is very readily activated by cephalin.

One of the most interesting of our results is the action of Witte peptone in causing a reappearance and preservation of thrombin in hemophiliac serum, and the activating action of cephalin on such serum-peptone mixtures. Peptone seems able here to correct the essential abnormality of hemophiliac blood. So far we cannot say in what manner peptone is able to exert such an effect. Neither have we tried any in vivo effects of peptone on hemophiliacs.

A second interesting point in this report is the evidence showing the development of normally reacting prothrombin in hemophiliac blood, along with the return of the clotting time to normal following the induction of a local protein skin reaction. This is of very great value clinically, since we may now keep our hemophiliacs sensitized to some protein, and either induce a local reaction every few weeks, or else wait until bleeding occurs to give the intradermal injection. This seems to us at present to be the most effective way of controlling bleeding in hemophiliacs. This reaction also interests us greatly, since it seems to form a valuable connecting link between the immune reactions and blood clotting.

SUMMARY OF RESULTS

1. Very fresh serum, obtained by clotting hemophiliac plasma with tissue fibrinogen, is found to contain a rich supply of active thrombin, but no prothrombin capable of activation by cephalin.

2. This thrombin very rapidly disappears from such serum, and at no time in the ageing process can any new thrombin be produced by cephalin such as we see in normal serum.

3. Cephalin in one case actually delays, and in the other case only very slightly accelerates the clotting of recalcified hemophiliac citrate plasma. This together with its inability to act on hemophiliac serum, leads us to believe that the fault lies in some fashion in the prothrombin factor.

4. There is found no increase in the antithrombin of hemophiliac blood or serum.

5. Protein sensitization and local skin reaction in a hemophiliac generates a normally reacting prothrombin in the blood and increasing the cephalin effect on the plasma and serum.

6. Tissue fibrinogen clots such blood equally well whether normal prothrombin be present or absent, confirming our views as to the two independent clotting processes.

7. Witte peptone preserves the thrombin of hemophiliac serum and enables cephalin to exert its characteristic action.

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EXPLANATION OF THE BLOOD CLOTTING CHANGE OBSERVED IN PEPTONE SHOCK IN THE DOG

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Much has been written about peptone shock in the dog but so far no satisfactory explanation of the blood clotting change has been set forth. Howell (1911), in his demonstration of an increased antithrombic content of such blood, has perhaps thrown the most light on the question, but even his work fails to account for the production of this antithrombin and its origin, as well as its exact manner of destroying the thrombin. In an accompanying article on the antithrombic action of serum proteins Mills (1925) demonstrates this action to be a cleavage of cephalin from its union with prothrombin (i.e., thrombin) causing the thrombin to disappear and prothrombin to be regenerated. This same action is carried on by the protein fraction of tissue fibrinogen, and seems in both cases to be dependent on the high cephalin combining power of these proteins, taking cephalin from its dissociable unions, such as thrombin, into a nondissociable combination.

It was undoubtedly this type of thrombin destruction that Howell was dealing with in his studies of the antithrombic activity of serum heated to 60°C. Since he (and others) have repeated shown that this antithrombic action is much increased in peptone plasma, we deemed it advisable to test by our method the thrombin splitting and prothrombin regenerating action of this plasma. As will be clearly shown below, serum from peptone plasma exhibits a greatly intensified thrombin splitting action, being in all other respects practically normal.

A dog of 12 kilos (not fasted) received 48 cc. of a 10 per cent solution of Witte peptone by rapid intravenous injection. The blood pressure exhibited the typical fall. A sample of blood was drawn from the femoral artery and citrated just before the injection. A second citrated sample was obtained 15 minutes after the injection, and a third sample (not citrated) was drawn 18 minutes after the injection. This last sample remained permanently fluid. All three samples were centrifuged and the plasma collected for use in the tests detailed below.

Spontaneous destruction of the thrombin in the serum of clotted pep-

tone plasma was first observed. Horse citrated plasma was used as the test fluid to measure thrombin action.

TABLE 1

PLASMA USED	ADDITIONS	AGE OF SERUM	TIME ELAPSING AFTER CEPHALIN ADDITION TO SERUM	1 PER CENT $CaCl_2$	CLOTTING TIME	REMARKS
				cc.		
3 cc. normal dog citrated plasma				0.3	1′20″	Serum saved for use below
0.5 cc. H.P.	0.25 cc. serum from above	3′			20″	
0.5 cc. H.P.	0.25 cc. serum from above	10′			50″	
0.5 cc. H.P.	0.25 cc. serum from above	25′			65″	
0.5 cc. H.P.	0.25 cc. serum + 1 drop C.	26′	1′		12″	
0.5 cc. H.P.	0.25 cc. serum + 1 drop C.	38′	12′		60″	
0.5 cc. H.P.	0.25 cc. serum + 1 drop C.		3 hrs.		4′20″	
3 cc. dog citrated peptone plasma				0.3	3′10″	Serum saved for use below
0.5 cc. H.P.	0.25 cc. serum from above	3′			30″	
0.5 cc. H.P.	0.25 cc. serum from above	12′			1′40″	
0.5 cc. H.P.	0.25 cc. serum from above	28′			2′	
0.5 cc. H.P.	0.25 cc. serum + 1 drop C.	29′	1′		30″	
0.5 cc. H.P.	0.25 cc. serum + 1 drop C.	41′	12′		2′30″	
0.5 cc. H.P.	0.25 cc. serum +1 drop C.	45′	16′		8′	
3 cc. peptone plasma not citrated	0.3 cc. tissue fibrinogen			0.3	1′	Serum saved for use below
0.5 cc. H.P.	0.25 cc. serum from above	1′			20″	
0.5 cc. H.P.	0.25 cc. serum from above	11′			6′	Very weak clot
0.5 cc. H.P.	0.25 cc. serum from above	25′				No clot
0.5 cc. H.P.	0.25 cc. serum + 1 drop C.	26′	1′		9″	
0.5 cc. H.P.	0.25 cc. serum + 1 drop C.	36′	10′		2′30″	Weak clot

H.P. = citrated horse plasma.
C. = 0.5 per cent emulsion of cephalin.

In table 1, we see that serum from normal dog citrated plasma clots horse plasma as follows:

When 3 minutes old it clots the plasma in 20 seconds.
When 10 minutes old it clots the plasma in 50 seconds.
When 25 minutes old it clots the plasma in 65 seconds.
One minute after activating it with cephalin it clots the plasma in 12 seconds.
Twelve minutes after activating it with cephalin it clots the plasma in 60 seconds.
Three hours after activating it with cephalin it clots the plasma in 4 minutes, 20 seconds.

Contrast these figures of 20 seconds, 50 seconds and 65 seconds before cephalin activation with the figures 30 seconds, 1 minute 40 seconds and 2 minutes 20 seconds obtained with the serum from the citrated peptone plasma and 20 seconds, 6 minutes and 8 obtained with the non-citrated sample. The difference is just as striking after cephalin addition to the aged serum. Thus the coagulation times with the normal serum were 12 seconds after 1 minute standing, and 60 seconds after 12 minutes standing, while the citrated peptone sample gave 30 seconds and 2 minutes 20 seconds after a corresponding period of standing and the non-citrated peptone sample 9 seconds and 2 minutes 30 seconds. This is a very striking difference in the rate of thrombin disappearance, both peptone samples destroying thrombin much more rapidly than the normal, but the second one of these being more active in this respect than the first (or citrated one).

Incidentally we bring out here that the prothrombin content of the peptone plasma is apparently quite normal and is entirely regenerated from the thrombin in the peptone serum. We see, for instance, in the last part of the table that after all thrombin has disappeared from the peptone serum in 25 minutes, the addition of cephalin causes a thrombin production sufficient to bring on clotting in 9 seconds.

It was noticed also in this last sample that the ability of the serum to destroy thrombin was somewhat less after the cephalin addition, probably because of the partial cephalin saturation of the serum proteins thus brought about reducing their tendency to disintegrate thrombin.

Having established that peptone plasma owes its non-coagulable property to an excess of thrombin-disintegrating elements (antithrombin of Howell) we next wished to be sure the injected peptone was not itself acting in this fashion. To this end we added the peptone solution to normal and peptone serum and observed the effect on the thrombin-splitting power of the serum. The normal rate of thrombin destruction in horse serum freshly activated by cephalin is shown in the first experiment. All clotting tests were carried out at 40°C.

Experiment 1. 3 cc. of fresh horse serum + 1 cc. water + 0.6 cc. of 0.5 per cent cephalin solution.

0.3 cc. of this serum mixture (1 minute 30 seconds after mixing) + 0.5 cc. citrated horse plasma clotted in 3 seconds.

0.3 cc. mixture (15 minutes after mixing) + 0.5 cc. citrated plasma clotted in 30 seconds.

0.3 cc. mixture (25 minutes after mixing) + 0.5 cc. citrated plasma clotted in 45 seconds.

0.3 cc. mixture (47 minutes after mixing) + 0.5 cc. citrated plasma clotted in 50 seconds.

0.3 cc. mixture (60 minutes after mixing) + 0.5 cc. citrated plasma clotted in 70 seconds.

0.3 cc. mixture (150 minutes after mixing) + 0.5 cc. citrated plasma clotted in 1 minute 30 seconds.

In one hour the thrombin activity of this serum degenerated from 3 to 70 seconds. Next was tried the same activated horse serum with the peptone solution added in place of water.

Experiment 2. 3 cc. fresh horse serum + 1 cc. of 10 per cent with peptone solution + 0.6 cc. 5 per cent cephalin solution.

0.3 cc. of this mixture (1½ minutes after mixing) + 0.5 cc. citrated horse plasma clotted in 3 seconds.

0.3 cc. of this mixture (15 minutes after mixing) + 0.5 cc. citrated horse plasma clotted in 7 seconds.

0.3 cc. of this mixture (25 minutes after mixing) + 0.5 cc. citrated horse plasma clotted in 15 seconds.

0.3 cc. of this mixture (47 minutes after mixing) + 0.5 cc. citrated horse plasma clotted in 35 seconds.

0.3 cc. of this mixture (60 minutes after mixing) + 0.5 cc. citrated horse plasma clotted in 44 seconds.

0.3 cc. of this mixture (150 minutes after mixing) + 0.5 cc. citrated horse plasma clotted in 1 minute 15 seconds.

We see strikingly brought out here that the peptone far from increasing the thrombin disintegration as does peptone plasma, exerts just the opposite effect, i.e., it markedly inhibits the normal thrombin destruction in horse serum. Thus, without peptone added, the thrombin activity of the serum is 3 seconds 1½ minutes after activation, 30 seconds after 15 minutes, 45 seconds after 25 minutes, etc., while with peptone present the figures are 3, 7, 15, 35, 44, 75 seconds, as contrasted with 3, 30, 45, 50, 70, 90 seconds above. It is to be noticed that the peptone effect is most marked in the first half-hour, lessening toward the end of an hour. A second similar set of tests was made, with the activity tested at more frequent intervals in the first half-hour. The control gave readings of:

4 seconds 1 minute after activation
40 seconds 11 minutes after activation
45 seconds 22 minutes after activation
50 seconds 32 minutes after activation
180 seconds 3½ hours after activation

The serum receiving the peptone, when activated with cephalin, gave

4 seconds 1 minute after activation
7 seconds 11 minutes after activation
15 seconds 22 minutes after activation
180 seconds 3¼ hours after activation

This brings out much more clearly the thrombin protection afforded by the peptone in the first half-hour, as well as the fact that this protection is lost on longer standing of the mixture.

Thinking that perhaps more peptone might give a more permanent thrombin protection we mixed equal volumes of freshly activated serum and 10 per cent Witte peptone solution, testing the thrombic activity as follows:

1 minute after mixing, clotting occurred in 20 seconds
2 minutes after mixing, clotting occurred in 15 seconds
3 minutes after mixing, clotting occurred in 10 seconds
9 minutes after mixing, clotting occurred in 10 seconds
29 minutes after mixing, clotting occurred in 15 seconds
43 minutes after mixing, clotting occurred in 15 seconds
60 minutes after mixing, clotting occurred in 19 seconds

Here is certainly a most striking thrombin preservation, when we compare these figures with those obtained from serum receiving no peptone addition. The effect of the larger amount of peptone is of much longer duration, also, than was that of the smaller amount. This fact will be discussed later.

Having demonstrated, now, that the *in vitro* action of peptone was just the opposite of that exerted by dog peptone serum, the next step was to see if peptone added to peptone plasma would reduce its excessive thrombolytic action just as it reduced the thrombolytic action of normal serum. A part of the final sample of peptone plasma mentioned earlier in this paper was clotted with tissue fibrinogen and the serum collected. The thrombin disappearance in this fresh serum was watched, with water addition as control, and with addition of 10 per cent peptone (1 cc. to 3 cc. of serum). The thrombin in the serum receiving only water disappeared as follows:

With the serum 3 minutes old it clotted plasma in 20 seconds.
With the serum 10 minutes old it clotted plasma in 60 seconds.
With the serum 26 minutes old it clotted plasma in 120 seconds.
One minute after now adding cephalin it clotted plasma in 12 seconds.

A very rapid thrombin destruction is seen to take place here. The last test, after cephalin addition, was merely inserted to demonstrate the regeneration of the prothrombin in this peptone plasma. It is to be compared to the figures given below for the same serum to which is added

peptone. To 3 cc. of the same fresh peptone serum used above was added 1 cc. of a 10 per cent Witte peptone solution and the following results of its thrombic activity obtained:

With the serum (+ peptone) 3 minutes old, it clotted horse plasma in 20 seconds.
With the serum (+ peptone) 10 minutes old, it clotted horse plasma in 25 seconds.
With the serum (+ peptone) 26 minutes old, it clotted horse plasma in 32 seconds.
One minute after now adding cephalin, it clotted horse plasma in 8 seconds.

Two facts are here evident: First the rapid thrombin destruction in the peptone serum is almost eliminated by the peptone added, and second, the presence of the peptone seems to increase the activation of prothrombin by cephalin (8 seconds below against 12 seconds above).

Peptone, *in vitro*, then, seems definitely able to counteract the change brought about in dog plasma by intravenous injection of this same peptone.

A second dog (weight, 20 kgm.) was fasted for 24 hours and then peptonized using 50 cc. of 10 per cent peptone solution. Six 20-cc. samples of blood were drawn from the femoral artery: *1*, before the peptone injection; *2*, 5 minutes after the injection; *3*, 10 minutes after: *4*, 15 minutes after; *5*, 20 minutes after; and *6*, 25 minutes after. All samples were drawn into sufficient 50 per cent sodium citrate solution to give a final concentration of 0.5 per cent. The samples were centrifuged and the clear citrated plasma used in the following experiments. They were first tested for ability to clot on simple recalcification. The first one, or control, clotted in 2 minutes on recalcification (at 40°C.), while none of the other five samples showed any evidence of clotting in 24 hours after receiving calcium. Evidently the peptonization was very thorough. A small amount of each plasma sample was heated slowly to 57° to remove the fibrinogen and was then tested for its thrombolytic power on activated horse serum. Using 0.5 cc. of the peptone plasma to 1 cc. of horse serum, the thrombin destruction was entirely too rapid to measure, so that on 0.05 cc. of each was added to the horse serum as indicated below. The horse serum (H.S.) received 0.2 cc. of 0.5 per cent of cephalin

Clotting time of 0.5 cc. horse citrated plasma by 0.3 of the following mixtures

STANDING FOR	1 cc. H.S. + 0.05 cc. H_2O	1 cc. H.S. + 0.05 cc. NO. 1	1 cc. H.S. + 0.05 cc. NO. 2	1 cc. H.S. + 0.05 cc. NO. 3	1 cc. H.S. + 0.05 cc. NO. 4	1 cc. H.S. + 0.05 cc. NO. 5	1 cc. H.S. + 0.05 cc. NO. 6
20″		10″	18″	15″	16″	14″	35″
1′	9″	10″	23″	20″	18″	16″	55″
3′	8″	13″	55″	40″	28″	26″	3′15″
12′	23″	60″	7′30″	10′	6′30″	9′	20′ trace
22′	45″						
70′	1′50″						

per cubic centimeter of serum one minute before adding the 0.05 cc. dog plasma. This gave a maximum amount of thrombin to be acted upon. A glance at this table of results and at the chart, demonstrates the very remarkable power of 1 drop of the heated peptone plasma samples to disintegrate the thrombin in 1 cc. of activated horse serum. The control sample (1), drawn before the peptone injection, increases somewhat the thrombin destruction as compared to the water control, but this is very slight as compared to samples 2, 3, 4, 5 and 6.

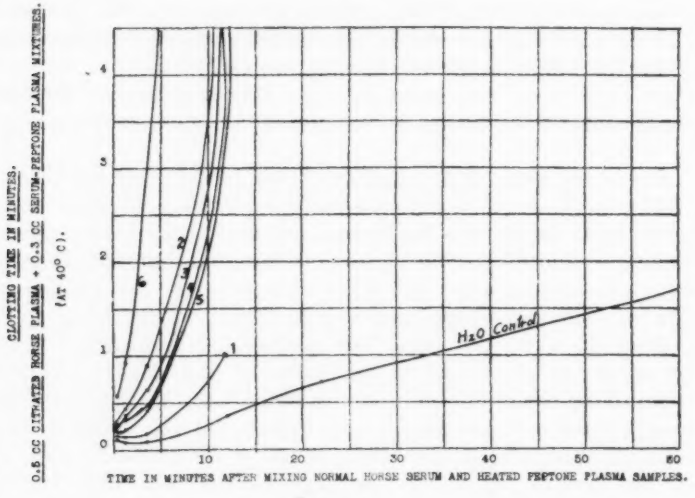

Fig. 1

To test again the ability of the peptone solution to neutralize the antithrombin of the peptone plasma, 0.05 cc. of the 10 per cent solution of Witte peptone was added to 0.05 cc. no. 6 and allowed to stand for 2 minutes. This mixture was then added to activated horse serum, as in the last table of results, and the rate of thrombin destruction observed.

Clotting time of 0.5 cc. horse citrated plasma by 0.3 cc. of the following mixtures:

STANDING FOR	1 cc. H.S. + 0.05 cc. H_2O	1 cc. H.S. + 0.05 cc. PEPTONE	1 cc. H.S. + (0.05 cc. PEPTONE + 0.05 cc. NO. 6	1 cc. H.S. + 0.05 cc. NO. 6
20″	11″	11″	11″	40″
1′	16″	11″	18″	1′15″
3′	20″	12″	22″	4′10″
15′	1′20″	50″	3′30″	22′

The peptone is seen to almost completely neutralize the thrombolytic action of sample 6, which was the most active one. This agrees with the result of the tests on the peptone plasma of the first dog.

Since peptone so thoroughly corrects the abnormal condition in peptone plasma, it should enable these samples, that did not clot at all on simple recalcification, to clot normally. This is just the result we obtained as shown in the following table.

TUBE NUMBER	PLASMA	10 PER CENT WITTE PEPTONE	CEPHALIN 0.5 PER CENT	$CaCl_2$ 1 PER CENT	CLOTTING TIME
1	0.5 cc. No. 2		0.2 cc.	0.1 cc.	24 hours
2	0.5 cc. No. 2	0.5 cc.	0.2 cc.	0.1 cc.	2 minutes
3	0.5 cc. No. 2	0.5 cc.		0.1 cc.	5 minutes
4	0.5 cc. No. 5		0.2 cc.	0.1 cc.	24 hours
5	0.5 cc. No. 5	0.5 cc.	0.2 cc.	0.1 cc.	4 minutes
6	0.5 cc. No. 5	0.5 cc.		0.1 cc.	10 minutes

The above tests clearly prove the corrective influence of peptone solution on dog peptone plasma. We might say that peptone exerts little or no influence on the clotting of normal recalcified plasma, and no effect whatever on tissue fibrinogen clotting. The serums from tubes 2 and 5, respectively, clotted 0.5 cc. horse citrated plasma in 10 seconds and 14 seconds, indicating a normal amount of prothrombin to be present, even though the plasma samples had stood in the ice box for 5 days. Evidently no union takes place between prothrombin of peptone plasma and the antithrombic proteins.

Discussion of results. Certain points are clearly demonstrated in this paper. 1. Peptone injection in the dog markedly *increases* the thrombolytic power of the dog's serum. 2. Peptone added to serum *in vitro* just as markedly *diminishes* the thrombolytic action of either normal serum or serum obtained from dog peptone plasma.

The significance of these points seems to us also to be quite clear. "Peptone" *in vitro* neutralizes the action of thrombolytic substances (we believe them to be proteins capable of taking cephalin into non-dissociable union), and it probably does this by itself combining with these substances, thus preventing their absorption of cephalin. Now, on injection of "peptone" intravenously, the same sort of union takes place, increasing the coagulability of the blood temporarily, but this is quickly followed by a negative clotting phase characterized by the outpouring of a great excess of these cephalin absorbing substances. The outpouring of them into the blood is the physiological response to their depletion through union with the peptone injected. Like all immune reactions studied, the production of the physiological antagonist of the injected substance continues for a

time after this substance is entirely neutralized. We believe, then, that we have here a typical immune reaction of the toxin-antitoxin variety; one which we have succeeded in analyzing to a considerable degree. This affords a possible approach to the study of immunity on a chemical basis.

SUMMARY OF RESULTS

1. Peptone injection in the dog calls forth a marked increase in the thrombolytic power of the dog's serum (i.e., the antithrombin of Howell).

2. Peptone added to serum *in vitro* just as markedly reduces the thrombolytic power of normal serum, and of serum from peptone plasma.

3. The response of the dog to peptone injection, with the excess production of bodies capable of uniting with the peptone, seems to be similar to such typical immune reactions as the toxin-antitoxin reaction, except that the time required is only a few minutes.

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THE MANNER OF ACTION OF THE PROTEIN FRACTION OF TISSUE FIBRINOGEN AS A BLOOD ANTICOAGULANT

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In a previous publication (Mills et al., 1923) we described a blood anticoagulant obtained from various body tissues. It was shown to consist of the protein fraction of tissue fibrinogen and to owe its anticoagulant action to its avidity for free cephalin. It was found to neutralize the coagulant power of tissue fibrinogen by combining with the free linkage on the tissue fibrinogen cephalin, thus preventing union through this cephalin with blood fibrinogen. This anticoagulant protein combined with cephalin to reproduce an active coagulant similar in all respects to the original tissue fibrinogen.

The above publication dealt only with the action of this protein anticoagulant on tissue fibrinogen clotting. In the last few months, since taking up the question of thrombin clotting, we have investigated the action of this substance anew with special reference to thrombin production and action. Briefly stated, these are the facts we have at hand to present: First, the anticoagulant very definitely and rapidly reduces the thrombic activity of serum; second, it does this by reconverting the thrombin back to prothrombin; third, it is without any apparent effect on the prothrombin itself; fourth, it inhibits the activation of prothrombin to thrombin; and fifth, all these phases of its action may be abolished by treating it with tissue fibrinogen (or cephalin).

We will present the experimental data on which these facts are based, and attempt to make clear just what changes take place. We believe that the entire action of the anticoagulant can be explained on the basis of its cephalin affinity, the dissociated cephalin of the serum being taken into a non-dissociated union with it, so that none is left for prothrombin activation. We also believe thrombin to be a loose union of cephalin and prothrombin, the cephalin of which may be extracted by the anticoagulant, thus destroying the thrombin and regenerating the prothrombin. Tissue fibrinogen unites with the anticoagulant as described in the previous publication, and effectively prevents its action on thrombin production.

1. Influence of the anticoagulant on preformed thrombin. The anticoagulant solution used throughout these experiments was prepared from fresh calf's liver. The liver tissue was hashed, rapidly dried in a warm air current, powdered and extracted thoroughly by benzene at room temperature. The fat-free tissue was then extracted with 0.9 per cent NaCl solution. This centrifuged saline extract was a very active inhibitor of clotting and was used in the experiments detailed. One-tenth cc. of it delayed the clotting of 1 cc. of recalcified citrate plasma for 24 hours, when it normally clotted in about 3 minutes. Citrated horse plasma (centrifuged) was used in all experiments where no special plasma was needed. The tissue fibrinogen used was a 1.5 per cent solution of the pure compound of 0.9 per cent NaCl, very kindly supplied us by the Wm. S. Merrell Company. It contained no free cephalin. The free cephalin used in certain experiments was a 0.5 per cent emulsion of cephalin purified from fresh calf brain.

To demonstrate the ability of the anticoagulant to disintegrate thrombin in serum the following series of tests were run.

Into each of 12 test tubes (kept in a water bath at 40°C.) was placed 0.2 cc. fresh active serum.

To the serum in each of 6 of the tubes was added 0.1 cc. of the antithrombin solution. Well shaken at once.

To the serum in each of the other 6 tubes was added 0.1 cc. of 0.9 per cent NaCl solution as control.

At the various time intervals below noted one of the tubes containing serum and anticoagulant, and one containing serum and saline, received 0.5 cc. citrated horse plasma (previously warmed to 40°C.). The clotting time of the mixture was noted.

MIXTURE STANDING AT 40°C. FOR	SERUM AND ANTITHROMBIN CLOTTED 0.5 CC. PLASMA IN	SERUM AND SALINE CLOTTED 0.5 CC. PLASMA IN
5″	13″	9″
15″	16″	9″
1′	25″	9″
5′	1′35″	20″
10′	2′30″	40″
28′	10′	3′

A similar series of tests was run again, except that the serum and antithrombin mixtures, and serum and saline mixtures were kept at room temperature, until time to add the plasma. The actual clotting tests were carried out at 40°C.

MIXTURE STANDING AT 18°C. FOR	SERUM AND ANTITHROMBIN CLOTTED 0.5 CC. PLASMA IN	SERUM AND SALINE CLOTTED 0.5 CC. PLASMA IN
5″	15″	10″
2′3″	19″	10″
4′	22″	10″
10′	55″	15″
20′	1′20″	19″
30′	1′35″	20″

These two series of tests clearly demonstrated the thrombin destroying action of the anticoagulant. The rate of thrombin disappearance in the anticoagulant mixtures was three to five times as rapid as in the saline control tubes. The thrombin disappearance was more rapid at 40°C. than at 18°C., but the antithrombic action of the anticoagulant was about the same at the two temperatures.

Since in these experiments we are dealing almost wholly with preformed thrombin, we feel the conclusion to be justified that the anticoagulant rapidly neutralizes the thrombic activity of serum. This may be brought about by union of the anticoagulant to the cephalin of thrombin (if there be cephalin in thrombin), or to deviation of such cephalin to the anticoagulant, in which case prothrombin should be found regenerated in the serum. This last is, in fact, just what occurred, as will now be shown.

2. Reconversion of thrombin to prothrombin by the anticoagulant. It is well known that the thrombin of serum is very unstable, disappearing rapidly from the solution. In following the curve of this thrombin disappearance we discovered a new fact of great importance. In serum freshly activated by cephalin practically all the prothrombin is converted to thrombin, but as this activated serum ages and the thrombin disappears, we found the prothrombin to be spontaneously regenerated, so that after about an hour both the thrombin and prothrombin content of the serum were almost the same as before the cephalin activation. This surprising discovery will be discussed in a separate paper on prothrombin. The point we wish to emphasize here is that such a spontaneous regeneration of prothrombin from thrombin does occur in serum.

In the following table of results, we show this disappearance of thrombin from freshly activated serum at room temperature, the thrombic activity of the serum being tested at the intervals noted by adding 0.5 cc. of it to 1 cc. of citrated plasma at 40°C. Thus, in 34 minutes the activity had declined so much that the plasma was clotted only after 360 seconds, whereas the serum, when freshly activated, clotted it in 40 seconds.

TIME	REMARKS	THROMBIC ACTIVITY OF ACTIVATED SERUM. CLOTTING TIME IN	THROMBIC ACTIVITY DEVELOPED ON A SECOND CEPHALIN ADDITION; OR A MEASURE OF PROTHROMBIN CONTENT. CLOTTING TIME IN	MEASURE OF PROTHROMBIN CONTENT AFTER TREATMENT WITH ANTICOAGULANT. CLOTTING TIME IN
11:27 a.m.	First cephalin activation of serum			
11:28 a.m.		40″	40″	40″
11:33 a.m.		60″	55″	40″
11:37 a.m.		180″	95″	40″
11:48 a.m.		300″	60″	40″
12:2 p.m.		360″	50″	40″
12:43 p.m.		300″	40″	40″

In the second column of results we show the time taken by 0.5 cc. of this same previously activated serum to clot 1 cc. of plasma, except here a second cephalin addition is made 1 minute before mixing with the plasma. This converts any unchanged prothrombin to thrombin. The first test made immediately after the first cephalin addition did not show any increased thrombic activity in the serum. As time passed, however, we saw that this second cephalin addition produced more and more additional thrombin above that remaining from the first activation. Finally, this second addition produced the original amount of new thrombin in the nearly inactive serum. To us, this indicates a spontaneous regeneration of prothrombin from the thrombin produced by the first cephalin addition. That this is a reconversion of prothrombin from thrombin, and not merely an activation of previously unchanged prothrombin, is proven by the fact that immediately after the first activation, addition of further cephalin produces no more thrombin. As soon as 6 minutes after the first activation, however, the second cephalin addition increases the thrombin content, so that the prothrombin regeneration comes on rather promptly. As the thrombin resulting from the first activation grows less and less, we see the effect of a second cephalin addition to become greater and greater, until finally the second activation produces just as great thrombic activity in the serum as did the first activation. We must infer, then, that cephalin addition causes practically all prothrombin to be converted to thrombin in short order, but that the reverse process begins in a few minutes and continues until practically all the prothrombin has been regenerated.

If, now, at any period in the course of this serum ageing, we add the anticoagulant to the activated serum, not only does the previously described destruction of thrombin take place, but we find the entire original amount of prothrombin to be regenerated. In order to show this several steps were necessary. After giving the anticoagulant 5 minutes in which thoroughly to remove all dissociated cephalin from the serum, the anti-

coagulant was itself removed by the addition of pure tissue fibrinogen. The result of this step was to leave the serum free of dissociated cephalin, but ready to receive such and not have it taken up by the anticoagulant. Cephalin addition at this time readily activated any prothrombin present, so that the thrombic activity developed might be taken as a measure of the prothrombin present. From the table of results given above, we see that at any time this treatment is applied to the serum the whole of the original prothrombin content is present. That is, cephalin addition to such treated serum always results in thrombin production sufficient to give clotting in 40 seconds, the same as did the serum just following the first activation.

The interpretation of the above phenomena throws interesting light on the question of thrombin. Prothrombin must form a loose union with cephalin (perhaps through calcium, since this element is necessary), a union from which cephalin is rather readily dissociated. Following cephalin activation, therefore, the other proteins of the plasma are constantly combining with the dissociated phospholipin, so that, as time goes on, more and more of the thrombin loses its cephalin to these other proteins, and the prothrombin is regenerated. We may thus again and again convert the prothrombin to thrombin and see it spontaneously return to its original state until the plasma proteins become saturated with the phospholipin. This view makes out thrombin itself as a very unstable compound, which may well account for the great difficulties in preparing and purifying it.

The action of the anticoagulant in converting thrombin back to prothrombin is, in all likelihood, merely a cephalin removal, as indicated above.

3. Stability of prothrombin in presence of the anticoagulant. Serum whose prothrombin content has been restored by the anticoagulant, may be kept for days, with only very slow deterioration of the prothrombin. Nor does any union seem to occur between the two substances. Removal of the anticoagulant by tissue fibrinogen at any time leaves the prothrombin with its original ease of activation.

4. Inhibition of thrombin production from prothrombin by the anticoagulant. In line with the work detailed above regarding the conversion of thrombin back to prothrombin, one would expect to find that the anticoagulant also prevents the initial activation of prothrombin. This is exactly what will be demonstrated now.

Citrated horse plasma was passed through a Berkefeld "V" filter to free it from tissue fibrinogen and free cephalin (cytozyme). It came through perfectly clear and transparent. It would then not clot (at 40°C.) for hours after simple recalcification, but would clot in a matter of minutes on cephalin addition together with recalcification. It clotted quite promptly

with tissue fibrinogen and calcium addition, or with active thrombin. It contains, then, blood fibrinogen and prothrombin, but no other free clotting factors.

As was stated above, this plasma will not clot on simple calcification, although it contains prothrombin capable of being activated. If pure cephalin be also added it clots solidly. We may conclude, then, that it contains no dissociated cephalin. If, now, this plasma be clotted by tissue fibrinogen (and calcification) the serum from such clotting is found to contain active thrombin. We have shown elsewhere that tissue fibrinogen is entirely incapable of prothrombin activation, its 40 per cent cephalin not being at all dissociated. The cephalin necessary for this thrombin production must, then, arise in the plasma itself after removal of the blood fibrinogen. If, now, this serum be added back to some of the citrated plasma (cephalin-free) clotting occurs, as shown below. But if it be added to the same plasma after recalcification, clotting occurs in about half the time. This can only mean that as the thrombin removes some of the blood fibrinogen, the prothrombin of the plasma is activated (calcium being present) and the clotting is carried on not only by the thrombin added, but by new thrombin produced in the clotting plasma. We must again assume a cephalin dissociation from some constituent present in order to explain the new thrombin production. (In another paper we are discussing the protective effect of blood fibrinogen on prothrombin, and the inability of thrombin production from the prothrombin to proceed until the prothrombin be released from the blood fibrinogen combination.)

We now tested the effect of the anticoagulant on thrombin production under the conditions just mentioned, where no free cephalin existed at the onset, but dissociation of it apparently occurred during the clotting process. The cephalin-free plasma was clotted with tissue fibrinogen (and recalcification) and the serum collected. Like portions of this serum were now added to similar amounts of the cephalin-free plasma; first, without recalcification of the plasma; second, with recalcification, and finally with recalcification and with the anticoagulant present. If the anticoagulant acts by deviating free cephalin to it, clotting in the recalcified plasma containing it should take place only as rapidly as in the uncalcified plasma. This is exactly what occurred, as seen from the experiment.

10 cc. cephalin-free citrated plasma + 1 cc. tissue fibrinogen + 2 cc. of 1 per cent $CaCl_2$—clotted.

The serum was collected and used below.

1 cc. of the same plasma + 00 cc. anticoagulant + 0.5 cc. serum—clotted in 40 seconds.

1 cc. of the same plasma + 0.2 cc. of 1 per cent $CaCl_2$ + 00 cc. anticoagulant + 0.5 cc. serum—clotted in 20 seconds.

1 cc. of the same plasma + 0.2 cc. of 1 per cent $CaCl_2$ + 0.1 cc. anticoagulant + 0.5 cc. serum—clotted in 40 seconds.

The presence of the anticoagulant here serves to prevent entirely additional prothrombin activation.

5. Removal of the inhibitory action of the anticoagulant by tissue fibrinogen. Tissue fibrinogen is known to unite with the anticoagulant through the extra linkage on the cephalin. If now, the anticoagulant be removed from the above pictures by addition of tissue fibrinogen and the prothrombin action then be seen to proceed normally, we might feel quite assured that the inhibition had been of the nature of cephalin deviation and had involved no real changes in the prothrombin itself. This was tested by repeating the last experiment with a slight change.

10 cc. cephalin-free citrated plasma + 1 cc. tissue fibrinogen + 2 cc. of 1 per cent $CaCl_2$ was allowed to clot, and the serum collected and used below.

1 cc. same plasma + 0.5 cc. serum—clotted in 40 seconds.

(1 cc. same plasma + 0.2 cc. of 1 per cent $CaCl_2$) +0.5 cc. serum—clotted in 20 seconds.

(1 cc. same plasma + 0.2 cc. of 1 per cent $CaCl_2$ + 0.1 cc. anticoagulant) + 0.5 cc. serum—clotted in 40 seconds.

(1 cc. same plasma + 0.2 cc. of 1 per cent $CaCl_2$ + 0.1 cc. anticoagulant); let stand for 2 minutes, then + 0.1 cc. tissue fibrinogen + 0.5 cc. serum—clotted in 20 seconds.

Thus we see the normal prothrombin activation to proceed as usual after removal of the anticoagulant, indicating that it has in no way affected the prothrombin itself by its presence.

The neutralizing action of tissue fibrinogen on the anticoagulant may be pointed out very plainly in another fashion. If the anticoagulant be added to a serum rich in thrombin and the thrombin content tested after only a few seconds, it is seen to be markedly diminished. If, however, tissue fibrinogen be mixed with the anticoagulant before adding it to the serum, no reduction in thrombic activity takes place.
Thus:

Tube 1—0.5 cc. fresh serum + 1 cc. citrated plasma—clotted in 44 seconds.

Tube 2—0.5 cc. serum + 0.25 cc. anticoagulant (agitated gently for 30 seconds) + 1 cc. citrated plsma—clotted in 65 seconds.

Tube 3—0.25 cc. anticoagulant + 0.5 cc. tissue fibrinogen agitated for 2 minutes, then added to 0.5 cc. same serum and agitated for 30 seconds + 1 cc. citrated plasma—clotted in 43 seconds.

Tube 4—0.5 cc. serum + 0.5 in tissue fibrinogen, agitated for 30 seconds, + 1 cc. citrated plasma—clotted in 45 seconds.

In tube 1, the untreated serum causes clotting of the citrated plasma in 44 seconds. Shaking this same serum with anticoagulant for 30 seconds reduces the thrombic activity so that clotting occurs only after 65 seconds, indicating a very considerable thrombin destruction in so short a time as

30 seconds. Previous treatment of the anticoagulant with tissue fibrinogen inhibits this thrombin destroying action, as is shown by clotting occurring in 43 seconds, as compared to the control of 44 seconds. The tissue fibrinogen alone is seen to have practically no action on the thrombin.

If the tissue fibrinogen-anticoagulant mixture be left mixed with the serum for several minutes, the reconversion of the thrombin to prothrombin by the anticoagulant comes into view again. This is to be accounted for on the assumption that the tissue fibrinogen, in uniting with the anticoagulant, does not completely satisfy its cephalin combining power, so that a slower deviation of cephalin from the thrombin to the anticoagulant still goes on.

Discussion of results. We found earlier that a study of the mechanism of the anticoagulant action of this protein on tissue fibrinogen clotting gave us a much clearer insight into the true nature of that type of fibrin formation. In similar fashion has a study of its action on thrombin clotting shed light on the nature of this clotting in no uncertain way. The experiments are clear-cut and decisive, leaving little room for doubt as to the interpretation. Thus by the use of this valuable cephalin combining agent we go step by step from thrombin destruction in a few seconds, to complete regeneration of the prothrombin from thrombin in a few minutes, and to the prevention of prothrombin activation into thrombin. In all likelihood, the effects of the anticoagulant in every case depend on its avidity for cephalin and its holding in a nondissociable form the cephalin it takes up. Tissue fibrinogen, by virtue of the cephalin it contains, unites with the anticoagulant, thus reducing its ability to take up free cephalin, and thus destroying its above mentioned effects on thrombin and prothrombin.

Concerning the great light shed on thrombin and its production by these experiments, a more complete discussion will be given in another paper on prothrombin and thrombin. Brief mention may well be made here, however, of the chief points of interest.

The destruction of thrombic activity with a prompt regeneration of prothrombin we think points clearly to the conclusion that thrombin is a loose union of prothrombin with cephalin (whether containing calcium or not we cannot say), and that any agent taking up cephalin, in a nondissociated state is able to reverse the thrombin formation and regenerate prothrombin. Apparently the other plasma proteins are able to thus bind cephalin somewhat, since serum activated with cephalin goes through a cycle of conversion of the prothrombin to thrombin followed by regeneration of the prothrombin. Presumably one could repeat that cycle until a saturation point for cephalin were reached, at which point thrombin should be more stable.

The lack of any effect of the tissue fibrinogen on thrombin formation and

action, renders it a very valuable agent for removing the anticoagulant from the scene of action. By the aid of these two substances, we believe we have considerably clarified the thrombin problem.

SUMMARY OF RESULTS AND CONCLUSION

1. The thrombic activity of serum is very rapidly and markedly reduced by the anticoagulant.

2. Activation of serum by cephalin converts all the prothrombin to thrombin. As this activated serum ages, the thrombin is gradually reconverted back to prothrombin spontaneously. This reconversion may be hastened and made complete at any time by adding the anticoagulant.

3. Addition of the anticoagulant to serum stabilizes its prothrombin, reducing the deterioration that normally occurs from day to day.

4. The presence of the anticoagulant prevents the conversion of prothrombin to thrombin.

5. Tissue fibrinogen added to the anticoagulant unites with it and prevents all the effects attributed to it above.

6. The anticoagulant in all instances acts to bind cephalin in a nondissociated form, thus freeing any medium to which it is added to its dissociable cephalin.

7. Since the anticoagulant acts thus, its destruction of thrombin must mean that thrombin represents a dissociable union between prothrombin and cephalin. The spontaneous regeneration of the former from the latter in serum is brought about by the serum proteins acting to bind the dissociated cephalin just as does the anticoagulant.

8. The rapid reconversion of thrombin to prothrombin by this cephalin absorbing compound gives distinct proof of the nature of thrombin. The stabilizing of the prothrombin is brought about by the anticoagulant keeping the serum free of dissociated cephalin.

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STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN BIRDS

XXI. Blood Calcium Changes in the Reproductive Cycle

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Sex studies being made by the senior author require information concerning the gross metabolic changes which may occur coincident with ovulation in pigeons; and also concerning the extent to which these changes are sustained when ovulations are "forced" or made to occur in rapid succession—a condition earlier found to be associated with profound changes in sexuality and in the sex ratio in pigeons. Facts already known suggest that the search for such gross metabolic changes is more likely to succeed if the glands of internal secretion are made the objects of investigation, since it is becoming clear that these organs are peculiarly and very intimately concerned in the phenomena of reproduction and of sex. Finally, the processes of reproduction are more accessible to study in birds than in any other class of vertebrates; and the applicability to mammals of the essential facts learned from the bird is highly probable, since mammalian reproductive processes have evolved from the type of reproduction still present in the bird.

The recent studies of Salvesen (1923), Collip (1925) and others have made it clear that normally the calcium level of the blood of mammals is under the control of a secretion of the parathyroids. Measurement of the blood calcium in the various stages of the reproductive cycle becomes therefore a practical means of obtaining information concerning a possible relationship of the parathyroids to reproduction. The various phases of the reproductive cycle are easily and certainly identifiable in pigeons, and for this reason the pigeon is more suitable than are other birds for such a study. The fact that the bird has a "calcium gland" (the "shell gland," or lower oviduct) which is brought into activity at a definite stage of the reproductive cycle will be found to constitute no proper objection to an application to mammals of the relation which the parathyroids bear to reproduction.

The outstanding result of the present study is the finding and definite establishment of the fact that the blood calcium is enormously increased —to more than twice the normal value—at the time eggs are ready to

leave the ovary. The active secretion of an egg-shell begins only about 15 hours after the egg leaves the ovary; but the beginning of the increase in the concentration of calcium in the blood dates from approximately 108 hours before ovulation—or a total of 123 hours before the egg-shell begins to form. These periods of beginning and of maximum increase of serum calcium thus prove to be quite the same as the periods earlier found for the hypertrophy of the suprarenals (Riddle, 1923) and for the rise of blood sugar (Riddle and Honeywell, 1923) at ovulation. A minor result of this study is concerned with evidence that the blood calcium undergoes a seasonal change—increasing from lowest levels in winter to highest in summer. The season of increased blood calcium is also accompanied in pigeons by decreased thyroid size and by increased size of testis and ovary.

PRESENTATION OF DATA. Common pigeons (Columba) and ring doves (Streptopelia) were studied during nine months—December to September. The diet and degree of confinement were uniform throughout the year. In the main study adult mated birds were used, and male and female mates were usually examined on the same day. Identical amounts of sunlight were afforded the two sexes, but not all mated pairs had equally lighted cages. The blood was obtained by decapitation; careful autopsies were done at once on every bird, thus making it possible positively to identify the reproductive stage and to classify separately the values found for diseased birds. The Kramer-Tisdall method of calcium estimation was used with a single slight modification.[1] When as much as 4 cc. plasma could be obtained determinations were usually made in duplicate; samples of less than 1.5 cc. were never used.

Blood calcium and the reproductive cycle. The plasma calcium values obtained for two kinds of adult pigeons at thirteen stages of the reproductive cycle are shown in table 1 and figure 1. Values for males and females are of course separately recorded, individual males being assigned to the same stage of the reproductive cycle as was made evident in their female mates. A comparison of the values found for common pigeons and for ring doves seems to justify the conclusion that although those birds belong to two different zoölogical families their blood calcium level is not obviously different. The values obtained for 191 healthy individuals of the two groups have therefore been combined in the bottom section of table 1,

[1] Dr. P. R. Wiener, of the Department of Metabolism, Vanderbilt Clinic, New York, kindly suggested the advantage of using 2 drops of ammonia to prevent the precipitation of Mg, instead of later using ammonia water to wash Mg from the precipitate. In a former study (Riddle and Honeywell, 1925), in which some very high calcium values were found in reproducing females, the above precaution was not taken and it was considered probable that the prescribed washing had not removed all Mg from the precipitate.

TABLE 1

Serum calcium (milligrams per 100 cc.) in the sexes in various parts of the reproductive cycle

CLASSES		MATURE BIRDS; RESTING STAGE	PRE-OVULATION PERIOD				DURING OVULATION	POST-OVULATION PERIOD			INCUBATION			FEEDING YOUNG
			108–73 hours	72–37 hours	36–20 hours	12–0 hours		0–36 hours	36–72 hours	73–108 hours	Early 1–4 days	Middle 5–9 days	Late 10–18 days	
Ring doves (healthy)														
Females	Calcium	9.5	14.2	14.7	19.3	18.2	19.9	11.7	9.6	8.7	10.3	9.3	10.2	9.3
	Number	9	2	2	7	5	6	2	3	4	6	5	6	3
Males	Calcium	8.8	8.4	9.0	8.8	9.8	9.1	8.3	8.9	8.1	8.7	9.1	9.5	8.6
	Number	16	2	2	6	4	6	1	3	2	7	7	6	3
Diseased														
Females	Calcium	9.7			22.0	13.1		21.0*					9.1	10.2
	Number	10			1	1		1					3	2
Males	Calcium	8.6	47.2†										9.0	
	Number	8	1										1	
Common pigeons (healthy)														
Females	Calcium	8.9	10.0	12.0	19.8	20.7		17.7	13.7	15.4		8.8	9.9	9.7
	Number	4	1	2	3	2		4	4	1		3	4	2
Males	Calcium	9.2	8.8	9.2	9.9	8.9	9.0	10.0	9.4			9.1	9.6	9.4
	Number	8	2	1	3	2	1	3	4			4	5	3

Diseased													
Females	Calcium	9.6	13.6		35.3			13.2			10.2	10.2		
	Number	3	1		1			1			2	1		
Males	Calcium	8.9									10.0			
	Number	2									1			
Ring dove and common pigeon values combined (healthy birds)														
Females	Calcium	9.3	12.8	13.4	19.5	18.9	19.9	15.7	11.9	10.1	10.3	9.1	10.1	9.4
	Number	13	3	4	10	7	6	6	7	5	6	8	10	5
Males	Calcium	9.0	8.6	9.0	9.2	9.5	9.1	9.6	9.2	8.1	8.7	9.2	9.6	9.0
	Number	24	4	3	9	6	7	4	7	2	**7**	11	11	6

* An ovum free in body cavity, oviduct small; time of ovulation uncertain.

† A mature (14.2 mo.) unmated male with eye "canker." No other similar case examined.

and in figure 2. With rare exceptions the 42 additional diseased adults of table 1 confirm the values obtained for healthy birds.

The table and the figures make it clear that the males of both groups have approximately 9.0 mgm. calcium per 100 cc. of blood, and that this value remains essentially unchanged throughout the various periods of the reproductive cycle. In both groups of females, however, a far different result is obtained; although the values for non-ovulation stages are quite similar to those for males it is found that at or near the middle of the "ovulation period" the females show an increase of the blood calcium to rather more than twice its normal value. It may be noted that the "resting period," as used in figures 1 and 2, is of indeterminate duration—days or weeks—and the space accorded it on the curves is relatively

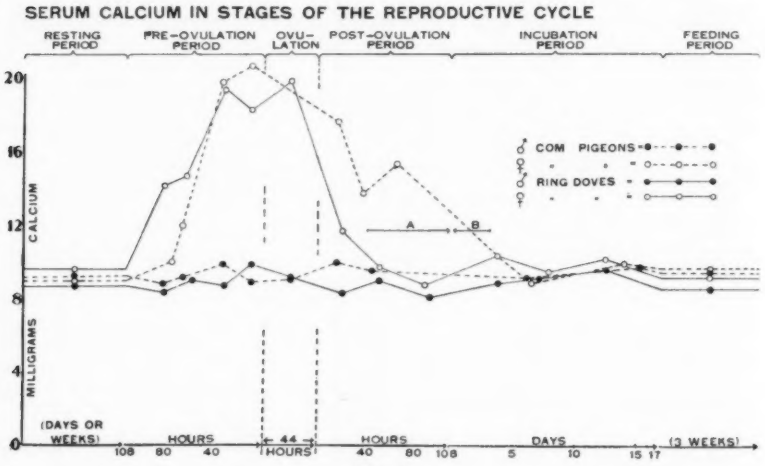

Fig. 1

much too short. The same thing is true of the "feeding period," the duration of which is 3 to 4 weeks. If the various sections of the curves could be drawn to a uniform and fairly typical time-scale the increased calcium values of the various ovulation (pre-ovulation to post-ovulation) periods would be limited to only about one-fourth or one-fifth of the entire reproductive cycle. On the scale actually adopted these periods occupy one-half of the entire cycle.

Two minor points connected with figures 1 and 2 require mention. The arrows labelled *A*, *B*, indicate a region of over-lap of time. Birds which were not permitted to incubate eggs following ovulation, and thus soon entered into a *resting period*, are correctly placed in region *A;* but those birds which were (during 3 days) simultaneously in *post-ovulation* and

incubation periods are plotted as belonging in the *incubation period* only. It is this circumstance that is chiefly responsible for the asymmetry of the right limb of the curves for females. In figure 2 the brackets (⌒) labelled *1st* and *2nd*, under *periods of shell secretion*, are intended to show by means of their position and length—the exact time of the secretion of egg-shell in relation to all other parts of the reproductive cycle. It will be noted that the beginning of shell formation on the first of a pair of eggs is approximately 123 hours removed from the time of beginning increase of calcium in the female blood.

Blood calcium, sex and season. An effort was made to secure enough data to throw light on the question of possible sex differences in the blood calcium. It would seem possible to use values obtained from adults

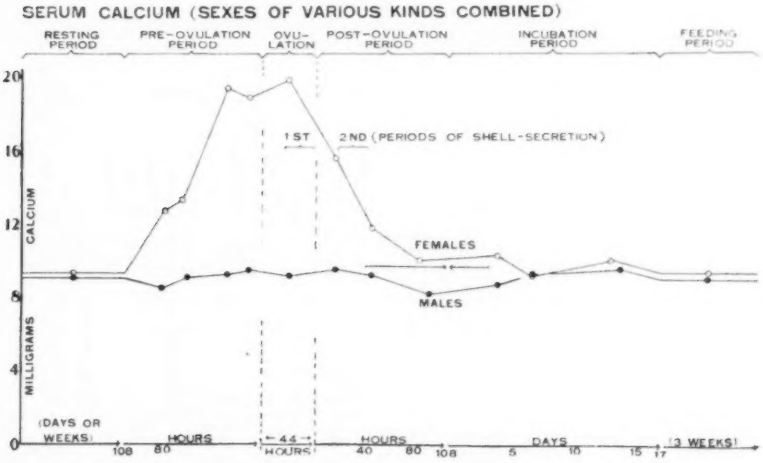

Fig. 2

in the "resting period" for such a sex comparison; but we also wished to have additional data from immature birds—a stage in which secondary effects of reproduction could not appear as sexual difference. For this purpose birds aged 3 to 6 months were killed at intervals during the year. The values for 47 juvenile birds thus studied, and the data for the "resting period" of adults, are recorded in table 2. Data for other periods listed in table 1 may also be examined. It will be seen that no consistent sex difference is found in our data. The data for the immature birds alone might suggest higher calcium values in the males, but the effect of season on blood calcium, to be considered immediately below, has been found to account adequately for this suggestion of a sexual difference in immature birds. It happens that a majority of the immature birds killed during

December-March (period of low calcium in healthy adults) were females, while those killed April-August were mostly males.

The relation of season to the blood calcium of healthy adult ring doves of both sexes is shown in figure 3. For the construction of these curves we seem justified (see table 1) in using data obtained from the "resting" and "feeding" periods, and after 5 or more days of incubation. The values are plotted by months. One finds a fairly distinct increase of blood calcium in spring and summer over the values obtained in winter. The number of determinations available (our common pigeons provide numbers too small for use) for a study of seasonal change is perhaps too small to make it entirely certain that this change actually occurs. On the same figure we have plotted the weights of the thyroids of the particular birds

TABLE 2

Serum calcium and sex (in juvenile birds and "resting period" only of adults)

KIND OF PIGEON	SEX	ADULT				JUVENILE				GRAND AVERAGE FOR EACH SEX	
		Healthy	Number	Diseased	Number	Healthy	Number	Diseased	Number	Ca	Number
Common pigeons	Male	9.2	8	8.9	2	8.8	3			8.98	13
	Female	8.9	4	9.6	3	8.6	3			8.82	10
Ring doves	Male	8.8	16	8.6*	8	9.8	19	8.5	1	9.20	44
	Female	9.5	9	9.7	10	9.0	15	9.6	6	9.38	40
Generic hybrids	Male	8.8	6							8.77	6
	Female	8.9	3							8.94	3
Totals	Male	8.9	30	8.7	10	9.6	22	8.5	1	9.11	63
	Female	8.9	16	9.7	13	9.0	18	9.6	6	9.19	53

* Including single bird with canker this value is 12.9.

which supplied the calcium values in the various months of the year. It will be seen that the thyroids tend to become smaller during those months (June excepted) characterized by increased calcium values. Reference to the more extensive data earlier obtained on this subject by Riddle and Fisher (1925) will show that the curve for thyroid size obtained from these 55 birds accurately represents (apart from the exceptional status of June) the normal seasonal change in the thyroids of pigeons. The data of figure 3 indicate that the season of apparent high blood calcium in pigeons is certainly the season of low thyroid size, and *vice versa*.

In figure 4 the curve for seasonal change of blood calcium is again re-

produced, and the size of the ovaries and testes of the particular birds which supplied the calcium data are plotted against the calcium curve.

Here it will be observed that periods of low blood calcium coincide with

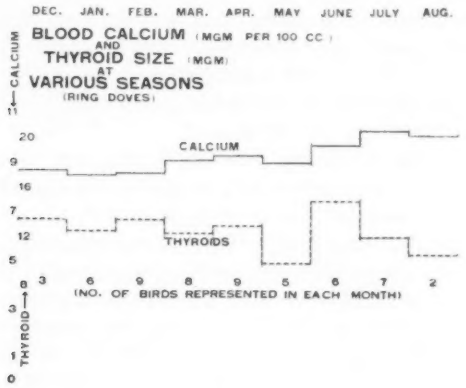

Fig. 3

periods of smaller size in ovary and in testis. Although the data given here for testis and ovary are rather inadequate, because of the considerable variability in size of these organs, an adequate amount of earlier data

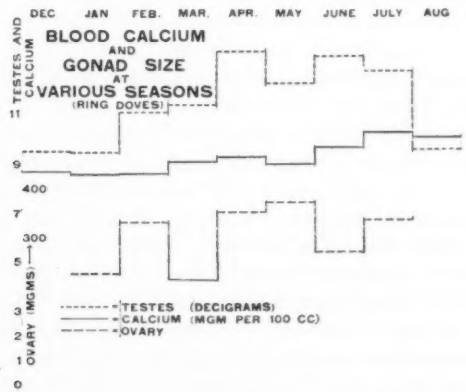

Fig. 4

(Riddle, 1925) shows that the seasonal change of ovary and testis which is indicated here is typical and normal.

It remains to point out that a few blood samples became cloudy or

showed hemolysis and that the values obtained from such samples have been excluded from all of the data presented above. There were 12 such samples upon which calcium determinations were actually made. In conformity with the experience of other workers, we find abnormally high values in practically all of these cases. From 3 healthy adults in the "resting period" an average value of 14.9 mgm. was found; from 5 such diseased adults, a value of 10.9 mgm. One healthy adult taken 10 hours before ovulation gave a value of 13.4 mgm.; 3 in "incubation" or "feeding" periods gave an average of 11.0 mgm.

DISCUSSION. The preceding data have a bearing on several points. For the first time in any animal it has been shown that a notable variation in the blood calcium level is coincident with *ovulation;* indeed this variation in the blood calcium is probably far greater than any other that ever occurs in the entire life of the bird, excepting only some cases of rare disease. One would therefore make inquiry concerning its occurrence in mammals; also whether human reproductive periods—menstruation, pregnancy, lactation, menopause—are known to be accompanied by changes of this kind. The further indication of seasonal changes in the calcium level of birds, with concurrent changes in some of the glands with internal secretion, suggests a comparison with any similar facts now known in other higher animals. A certain amount of data for a proper comparison of blood calcium in the two sexes has been presented. The bearing of possible changes in calcium metabolism (parathyroid activity) at ovulation upon the sex ratios obtained from pigeons subjected to continuous or forced ovulations supplied the reason for this study. These several topics require further consideration here.

This discussion should be preceded by a reference to four earlier observations on the blood calcium of birds. Blair Bell (1908) made a short series of observations on microscope slides (hematocytometer plates) of the number of calcium oxalate crystals precipitable from the blood of three hens—one not laying and two others actively laying eggs. The relative number of crystals was expressed in terms of an "index," and the blood of the birds was examined daily during one week. One hen laid eggs on alternate days and from the crystal-counts Bell concluded that in such a bird the blood calcium rises and falls daily "in preparation for the laying of an egg on the alternate day." His application of his findings was to parturition in mammals, and he makes no reference to their relation to ovulation. The crude method used is doubtless responsible for the chief fluctuations found and remarked upon by this observer; nevertheless, we may call attention—as Bell did not—to the circumstance that his counts from the non-laying hen average definitely lower than those from either of the two laying hens. On this point his observations coincide with the present results.

Steenbock, Hart, Jones and Black (1923) obtained (de Waard method) a value of 12.6 mgm. calcium per 100 cc. serum in six "control" fowls, and much larger values (16.9 and 22.6 mgm.) from fowls treated with cod liver oil. Ackerson, Blish and Mussehl (1925) obtained from the serum of 56 "control" fowls an average of 10.6 mgm.; from 51 rachitic chicks, an average of 7.5 mgm. Riddle and Honeywell (1925) made a series of observations on adult reproducing male and female pigeons, the sexes having been kept under identical conditions in respect of diet, sunlight, and degree of confinement. The average of 23 determinations of the serum calcium of male ring doves and common pigeons was 9.6 mgm. (range, 8.2 to 12.0); for 25 females the average was 12.1 mgm. (range, 8.9 to 18.9). Twenty additional determinations on pigeons of a peculiar hybrid type gave an average of 10.6, with values ranging from 8.7 to 12.5 mgm. It was concluded that the calcium values obtained from such adult reproducing females were higher than those from males, but that the difference observed was probably unduly influenced by a reproductive factor not always possible to exclude in those data. The present work makes it clear that such a reproductive—not a sex—factor is in fact essentially responsible for the difference found.

It is doubtless because of the uncertainty of the time of ovulation in human and most other mammalian forms that we are without knowledge of the relation of the blood calcium level to ovulation in these forms. It is true that Sherman, Gillett and Pope (1918) measured the daily calcium *excretion* of two sexually mature women during one month, and that no significant fluctuations were found. This result, however, does not exclude the existence of fluctuations in the blood calcium. There are many references in the literature to blood calcium measurements at one or another stage of the reproductive cycle, other than ovulation. Fluctuations at menstruation, pregnancy, lactation and menopause are considered in the several studies cited below.

Bell and Hick (1909), using methods that have been questioned by various workers, find that the menstrual blood itself is very high in calcium; that the blood calcium at first augments and then sharply falls before menstruation; that it rapidly decreases during, and rises to normal at the end of, this period. The method used (count of calcium oxalate crystals), however, also gave marked "diurnal variations" in the blood of normal persons. de Wesselow (1922) found smaller amounts of plasma calcium in the later stages of pregnancy. Leicher (1922) noted a fall of the calcium value after the menopause—45 to 54 years. Widdows (1922) found that calcium values obtained midway between the menstrual periods show no consistent difference from those obtained during menstruation. In a later study (1923) this observer reported a distinct tendency of the serum calcium to remain constant in early stages of preg-

nancy; to decrease in later stages; to return to normal directly after confinement; and further to increase during the early stages of lactation. Malamud and Mazzócco (1923) report higher calcium values (7.76 mgm. in 20 cases) in adult human females before the menopause than after this period (6.62 mgm. in 22 cases); they find lower values during lactation. Malamud (1924) comparing calcium values obtained on the whole blood of hospital patients several days before or after menstruation with those obtained on the first and last days of menstruation, reports wide variations at all periods with possibly a tendency to hypercalcemia at the end of the period. Blanchetiére (1925) states that an increase of calcium occurs after the menopause and after ovariectomy.

The foregoing reports are not in good agreement. Most of the better evidence seems to us to indicate that consistent variations have not been found at menstruation; also that a decrease of blood calcium does occur in the later months of pregnancy and at the menopause. Until daily blood calcium determinations—conducted during a month—are made on menstruating human subjects maintained under standard conditions, or until determinations are made in certain mammals whose ovulation time is known, it will not be possible to know whether a change occurs at ovulation in the blood calcium of mammals similar to that here found in birds. If such fluctuations do occur in mammals this fact may account for a part of the individual variation in the blood calcium values obtained from human and other females, and for the failure to find consistent calcium differences for menstrual and inter-menstrual periods.

As suggested in our introductory statement, it can not be assumed that the rise of blood calcium found in the pigeon is adequately accounted for by the circumstance that the bird will soon thereafter secrete and excrete a large amount of a calcium compound in the form of egg-shell. The available evidence indicates that an increase of the blood calcium is a regulated process in which the parathyroids probably play a predominant rôle; and that this increase begins days (approximately 123 hours) before egg-shell formation begins. Moreover, it is wholly fallacious to assume that the amount of blood calcium will be high at a time when unusual amounts of calcium are being removed from the blood for shell formation; it is quite as probable or even more probable, from *a priori* considerations, to be unusually low because of this drain of calcium from the blood into the lower oviduct (shell gland). As has been seen above, the maternal blood has a reduced calcium value during the later months of pregnancy when a heavier drain upon its calcium content is made by the bone-developing fetus. It may turn out that the increase of blood calcium at ovulation in mammals is small, or non-existent; in that case it would doubtless mean that this ancestral condition has been modified or lost in the evolution of mammals. Some, of course, of the lowest mam-

mals produce eggs not unlike those of birds. The observed fact—a most marked rise of a regulated constituent of the blood—must be considered in terms of the mechanism which produces this increase.

Our evidence for a seasonal change of the blood calcium level is somewhat strengthened by two earlier observations that have been made on seasonal change in blood phosphates and in the size of the parathyroids. The value of the seasonal change in phosphates as evidence lies in the circumstance that in healthy individuals blood phosphorus and blood calcium usually tend to change in the same direction. Hess and Lundagen (1922) found in the blood of infants a low percentage of phosphorus in winter and a high percentage in summer; and this effect is attributed mainly to the seasonal variation of sunlight. This is precisely the relationship that we find for season and calcium. Still more closely identified with the present topic are the results obtained on rabbits by Grant and Gates (1924) who noted a marked diminution of size in the parathyroids between November and February. Further, treatment of the animals with ultra-violet light produced more notable size changes in the parathyroids than in other organs—the parathyroids becoming markedly enlarged. Grant and Gates (1925) also carefully studied the blood calcium of the treated and untreated rabbits, but an effect of caging or confinement was found which served to complicate the data for seasonal change. These investigators have hesitated to draw a conclusion as to a seasonal change of blood calcium, and emphasize rather that "a parathyroid hypertrophy of 20 to 50 per cent does not result in a corresponding increase in the calcium content of the blood when the concentration is already at or near the normal level." They find that in general the blood calcium and phosphorus tend to change in the same direction.

Our attempt to measure possible differences in the blood calcium of the sexes has not met with success. The fact that a large increase of the calcium value occurs at ovulation makes it possible to use for this purpose only values obtained from non-ovulation stages of adult females; and the adequate demonstration of the actual reproductive stage is obtained only by killing the bird for examination at the time the blood sample is taken. The number of juvenile males and females which we took to supplement the data from adults turns out to be too small to permit a conclusion. This failure became evident when it was found that season appears to have a much more pronounced effect than has any possible sex-difference, and that unequal numbers of male and female immature birds had been examined at the various seasons.

It is not assumed here that the parathyroids alone are concerned in the regulation of calcium metabolism in higher animals. The studies cited at the beginning of this paper, however, seem to us practically conclusive as to the predominant part played by the parathyroids in the

mobilization of calcium. Two or three facts connected with this matter require mention here. First, the circumstance that the *utilization* of calcium for the formation of egg-shell is also conditioned by the thymus of birds. The studies of Soli (1911) and Riddle (1924) leave little doubt on this point. Leites (1924) has further found that thymectomy in dogs results, after several days, in a fall of the blood calcium from about 12.0 to nearly 8.0 mgm. Again, Wheeler (1919) has shown that strontium can largely replace calcium for the formation of both bone and egg-shell in the fowl. Second, the utilization of calcium by the bones, and perhaps also the calcium level of the blood, are rather generally thought to be more or less affected by the action of still other incretory organs—notably by the ovaries. Much of the literature on osteomalacia deals with this last-named subject. The results of blood calcium determinations following ovariectomy have a direct bearing on this question, but the various reports are quite contradictory.

There may be more than coincidence in the relation which our data show concerning seasonal variation in the blood calcium on the one hand, and thyroid, testis and ovary on the other. Thyroid size is lowest when calcium is highest and when, from studies cited above, the parathyroids are probably largest. In this case also, therefore, the thyroid and parathyroid appear to have not parallel but reciprocal phases of activity. The gonads—both testis and ovary—increase in size parallel with the increase in blood calcium and parathyroid activity, and these seasonal increases are certainly accompanied by a decrease of thyroid size.

The very high levels attained by the blood calcium in pigeons are of some interest. Collip (1925) and Collip, Clark and Scott (1925) were at first inclined to think that large injections of parathyroid hormone may cause death in dogs as a result of the high blood calcium levels (15 to 21.5 mgm.) thus produced. More recently (Collip and Clark, 1925) this view was somewhat modified and it is now held that death ensues only after prolonged hypercalcemia. In our study we have encountered the following very high calcium values in healthy females at or near the ovulation period: 21.4, 24.6, 24.3, 20.5, 21.6, 23.3, 21.7, 27.2 mgm.; in such a diseased female, 35.3 mgm.; in a male affected with eye "canker," 47.2 mgm. Several of these determinations were done in duplicate (1 of 24.3 mgm., in triplicate), and in practically all these cases the male mate of the bird showed a normal calcium value. It therefore seems entirely clear that doves and pigeons withstand during three to five days, without trace of adverse affect, a degree of hypercalcemia far in excess of that which was found to accompany coma and death in dogs.

Attention may be called to the probable bearing which the facts here presented have on the disturbances frequently seen in parathyroidectomized mammals at one or another reproductive period. Some workers, notably

Luckhardt and Goldberg (1923), have called attention to the circumstance that their animals became tetanous at oestrus. It seems possible that at this period something in the mammal, as in the bird, calls for a higher level of blood calcium than at other times—that the failure of calcium to be thus adjusted at this period is of more serious consequence than at other periods. We already know, for example, that this period of high blood calcium in the pigeon is accompanied by enlarged suprarenals (Riddle, 1923) and an increased blood sugar (Riddle and Honeywell, 1923).

It remains to consider the application of the changes in blood calcium and of parathyroid activity to the problem of sex in pigeons. Whitman (1919) and Riddle (1916, and earlier and later papers) have shown that forced or continuous ovulation in the pigeon results in a marked modification of the sex ratio, of the degree of sexuality (according to Riddle), together with ultimate effects on longevity and viability of the offspring. It is necessary to investigate the mechanisms by which these effects are produced. A prolonged study by the senior author on the changes which occur in the ova of pigeons under such conditions of crowded reproduction leads to the conclusion that the rate of metabolism within these ova is notably affected; and that this metabolic change is responsible for the observed changes in sexuality, longevity and viability shown by the offspring which develop from the ova thus modified. In further test of this view one wishes to know whether, and which, gross metabolic changes occur in the soma or body in association with these metabolic changes in the ovum or germ. The fact that certain aspects of reproduction in the pigeon have been found closely bound up with sexuality, and with metabolic level in the germ, together with the fact that the various endocrine glands play a leading part in the regulation of the various aspects of metabolism, make it necessary to examine several aspects of reproduction and all of the glands of internal secretion in their relation to reproduction and to sex in order that a comprehensive knowledge of the physiology of sexuality may be obtained.

As elements in the above-mentioned general program earlier studies of the glands of internal secretion of the pigeon have already been made on the ovary, the suprarenal cortex and medulla, and in part upon the thyroid. In the present study the attempt has been made to learn the relation of the parathyroids to ovulation—and thus their rôle in continuous or forced ovulations. The work was undertaken in the autumn of 1924. At that time the work of Salvesen (1923) and others seemed to give a sufficient basis for the study of parathyroid activity by means of measurements of the blood calcium. Meanwhile the studies of Collip and coworkers have appeared, and the admirable results of their work make it quite clear that under normal conditions blood calcium values are chiefly

an expression of parathyroid activity. In advance of Collip's work it was uncertain that supernormal calcium values are ascribable to the parathyroids. At present it seems safe to conclude that the very high calcium values found to accompany each ovulation period in the pigeon is an expression of increased parathyroid activity.

Under conditions of continuous reproduction (ovulations) it therefore becomes evident that the parathyroids are kept in practically continuous functioning at an abnormally high level. Under these conditions the blood calcium of a female forced through an ovulation period during each week or 10 days would practically never be permitted to fall to the normal level. This result may best be pictured by reference to figure 2. The calcium rises during a period of about 4½ days, remains at its height for nearly two days, and requires an additional 4 to 5 days to return to normal. In forced reproduction (i.e., when a new ovum is permitted or forced to begin its growth immediately after a second egg is ovulated) the rise in the calcium value for the next prospective ovulation is largely or entirely completed before the fall from the preceding ovulation has been accomplished. Thus the animal under forced reproduction is kept almost or quite continuously at a high (parathyroid) calcium level, whereas the pigeon under normal reproduction passes not more than one-fourth to one-tenth of her time under similar conditions. Under such "forced" reproduction an increasing predominance of female offspring results. In this modification of sexuality it is found that season, directly or indirectly is involved.

The increase and decrease in blood calcium at ovulation are apparently quite coterminous with the previously described increase and decrease of size in the oviduct and suprarenal; with increase and decrease of the blood sugar; and with the increase in size of the particular ovum which will ovulate 108 hours later. The seasonal rise of calcium is coterminous with largest size of testis and ovary; with smallest thyroid size in the parent; and with the greatest excess of females in the offspring. It has thus been made evident that the parathyroids play a part in reproduction, and with that particular phase of reproduction which has earlier been shown to be involved in experimental modifications of sex.

SUMMARY

The relation of the parathyroids to reproduction has been studied by means of measures of the serum calcium of male and female pigeons kept under constant conditions of food and confinement. Data were obtained for thirteen different stages of the reproductive cycle. A rôle of the parathyroids in avian reproduction has been found and described.

Male doves and pigeons show no marked fluctuations of the serum calcium level in the various stages of the cycle. Female pigeons regularly

show an extremely large increase in blood calcium—to double the normal value—at each ovulation period.

This rise of blood calcium in the female begins approximately 123 hours before the beginning of the formation of an egg-shell, and is in no wise explained by a large calcium "need" for shell formation. It is probably first of all an expression of a newly found relation which the parathyroids bear to reproduction in the female bird.

During three to five days female pigeons withstand, without trace of adverse effect, a degree of hypercalcemia considerably in excess of that thought by Collip and co-workers to result in death in dogs.

The available evidence for blood calcium changes during menstruation, pregnancy, lactation and menopause in the human and other mammals is discussed.

Evidence for a seasonal change in the level of blood calcium is found. In immature pigeons lower values were found in winter and higher values in summer. In this same animal it is known that the testes and ovaries undergo a size change in the same direction as the serum calcium, and that the thyroid is largest at the time the serum calcium is at its minimal seasonal value.

Insufficient and inconclusive data were obtained on the question of a sex difference in the amount of calcium in the blood.

These studies were made in continuation of an extended examination of the mechanisms by which forced or crowded ovulations effect modifications of sexuality and sex ratios in pigeons. It is found that under the conditions which bring about those sexual changes the parathyroid activity—as measured by the serum calcium—is maintained at an abnormally high level.

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THE RABBIT METHOD OF STANDARDIZING INSULIN[1]

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Several methods of determining the potency of insulin preparations have been proposed, based upon the physiological response of different species of small animals. The rabbit was the first animal used and it continues to be the one most often chosen for this work.

Banting, Best, Collip, Macleod and Noble (1) discovered that if a sufficient quantity of insulin is injected subcutaneously into a rabbit, the blood sugar is lowered from the normal level of about 0.10 per cent to about 0.045 per cent. At this latter level, characteristic symptoms of insulin overdosage were observed. The original definition of a unit was that amount of insulin necessary to lower the blood sugar of a fasting rabbit weighing 2 kilograms to the convulsion level of 0.045 per cent.

Two methods are in use for standardizing commercial insulin. The first of these is the official method used by the Connaught Antitoxin Laboratories of the University of Toronto. This was devised by Macleod and Orr (2). The average decrease in blood sugar over a period of 5 hours after the subcutaneous injection of insulin is taken as the criterion of potency. The second method is that used by Eli Lilly & Company. This standardization is based upon the incidence of convulsions in a group of several hundred rabbits injected with graded doses of insulin. The unit dose is considered to be the smallest amount required to produce convulsions in 75 per cent of the animals. In both of these methods, the rabbits are fed mixed diets and are fasted for 24 hours before the test. The injections are given subcutaneously. It is to be specially noted that the first method uses the decrease in blood sugar as the measure of potency, while the second method is based upon the production of convulsions.

In a paper from this laboratory (3) it was shown that rabbits fed a high carbohydrate, acid-forming diet were more resistant to insulin than those maintained upon a low carbohydrate, base-forming diet. The fact was stated that animals which have never received insulin are more resistant than those which are accustomed to it. We also found that the dosage of

[1] This work was supported in part by a special insulin grant from the Carnegie Corporation of New York.

insulin varies directly with the body weight. In view of these findings the following procedure has been followed in our laboratory: *a*, rabbits are fed a simple, standard diet of alfalfa hay with the addition of crushed barley after the production of convulsions and the following day; *b*, the rabbits are never used for accurate assay until they have become "educated" to insulin; *c*, the animals are used every 7th day and are always given a convulsion on that day; *d*, growing rabbits must show satisfactory gains in weight and mature animals must maintain their weights; *e*, a complete 24 hour fast precedes the test; *f*, farrow females are used; and *g*, the insulin is given intravenously in such dilutions that about 0.5 cc. contains a kilo convulsion dose. The above scheme controls to a large extent factors which cause variations in resistance of rabbits to insulin. These regulated factors are: variations due to diet; variations due to sex; and variations due to lack of "education." Discarding all animals which show disease of any kind controls another variable. Differences in resistance due to climatic changes do not affect us appreciably because of our equable climate. It was hoped that a close adherence to the above schedules would give an entirely satisfactory standardization of insulin. We have found, however, that other variables are not controlled by the procedure.

During the course of our experience with the standardization of insulin covering a period of more than three years, using an average of 6 rabbits daily the first two years and an average of 12 rabbits daily the last year, we have noticed that different breeds react variously to insulin. White or partly white rabbits are very resistant. We have discontinued the use of all except the Flemish and New Zealand breeds. Rabbits of these two breeds show a much more uniform reaction to insulin, but a considerable discrepancy between individuals still remains.

The fact that rabbits maintained under the above rigid plan respond differently to insulin indicates that an accurate assay cannot readily be made using such animals chosen at random. It was noted that individual rabbits reacted to a given minimum dose of the same insulin preparation week after week, with an error of $\pm$ 10 per cent. We determined, therefore, to select a certain preparation of insulin as a standard and to standardize our rabbits with respect to it. In this way, we believed that an accurate assay could be made by the use of a few standardized animals. For this purpose our insulin no. 283 was chosen as the standard. Its strength was determined to be 83 units per cubic centimeter when using a reliable rabbit, no. 6. We then selected 16 rabbits of different weights, some of which had been used for more than a year and none for less than four months. One cubic centimeter of the standard insulin was diluted to 50 cc. and 0.7 cc. of this dilution per kilo was injected. This dose represented a little over one unit per kilo. The same dose was repeated the following week in the case of the rabbits which convulsed, and was in-

creased to 0.8 cc. per kilo for those that did not show symptoms. A week later the rabbits that convulsed on the first dose received 0.10 cc. per kilo less. This dosage was maintained for a period of several weeks with determinations of the blood sugar before and after the insulin was given. This process was continued until the amounts of insulin required to produce convulsions in all of the animals had been determined. During these experiments the following observations were made: a rabbit that convulsed with a minimum dose repeatedly did so upon receiving the same amount; and a rabbit that convulsed within a given period of time on a certain minimum dose did so consistently. As a result of this study, we were able to assign to each rabbit what we are pleased to call a factor of resistance. This factor is obtained from the equation $\frac{Q}{SQ} = F$, in which Q

TABLE 1

Standardization of rabbit 73

DATE	WEIGHT	DOSE PER KILOGRAM	UNITS PER CUBIC CENTIMETER	FACTOR OF RESISTANCE	TIME TO CONVULSION	BLOOD SUGAR		
						Before	After	Difference
1925	*kgm.*	*cc.*			*minutes*	*mgm. per 100 cc.*		
July 22	2.83	0.7	71		74			
July 29	3.00	0.6	83		90			
August 5	2.95	0.6	83					
August 12	2.90	0.7	71		75	121	35	87
August 19	3.03	0.6	83		S.C.*	125	37	88
August 26	2.95	0.65	77	1.16	92	105	38	67
September 2	3.03	0.65	77	1.16	75	111	26	85

Insulin no. 283, dilution 1:50.

* Symptoms for 2 hours. No convulsion.

represents the minimum quantity of standard insulin necessary to produce convulsion and SQ represents the standard quantity of standard insulin containing one unit. Thus rabbit 13 convulsed repeatedly on 0.5 cc. per kilo of a 1:50 dilution of standard insulin 283. Its factor of resistance is, therefore, $\frac{0.50}{0.60} = 0.83$. Rabbit 90 convulsed on a minimum dose of 0.65 cc. of the same material and its factor is $\frac{0.65}{0.60} = 1.08$. When the potency of an insulin preparation is to be determined, the dose to be given to a standard rabbit is multiplied by its factor of resistance. Thus, suppose it is desired to give the equivalent of 0.5 cc. to a rabbit weighing 3.25 kilos whose factor is 0.85, the total dose to be injected is $0.5 \times 0.85 \times 3.25 = 1.3$ cc. This dose of 1.3 is equivalent to that of 1.6 cc. in the case of a rabbit of the same weight but having a factor of 1.0.

Table 1 illustrates the method of standardizing a rabbit and the results obtained. This animal always convulsed on doses of 0.65 cc. per kilo but failed to do so in two of three trials when 0.6 cc. was given. The factor was considered to be 1.08. Attention is called to the fact that the time to convulsion varied within the narrow limits of 75 to 92 minutes. The blood sugar determinations gave satisfactory values both before and after the injections.

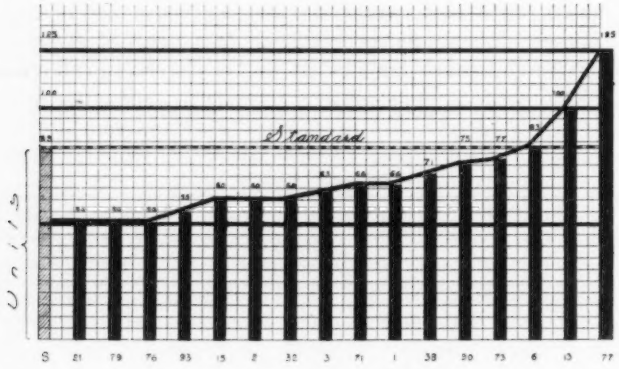

Fig. 1. Standardization of rabbits

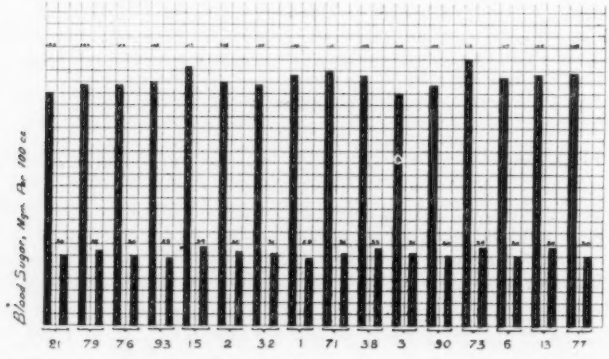

Fig. 2. Blood sugar of standard rabbits before the injection of insulin and immediately before the production of convulsions.

Figure 1 shows the values of our standard insulin no. 283 for 16 rabbits. It will be seen that the indicated potency of this preparation varied from 50 to 125 units per cc. or from 40 per cent less to 50 per cent more than the standard value. This chart emphasizes the difficulty of making any ac-

curate assay without the use of a factor, even when the rabbits are kept under a rigid regime.

In figure 2 are given the average values for the blood sugar of these standard rabbits before the injection of insulin and just prior to the pro-

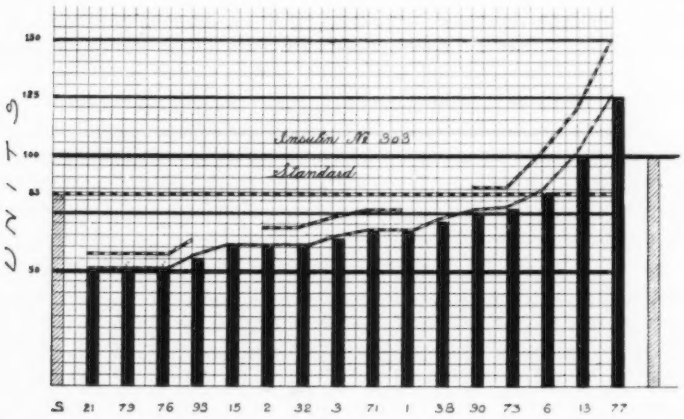

Fig. 3. The assay of an insulin preparation using factors of resistance. The parallelism of the two curves indicates that the standard rabbits react uniformly to different preparations.

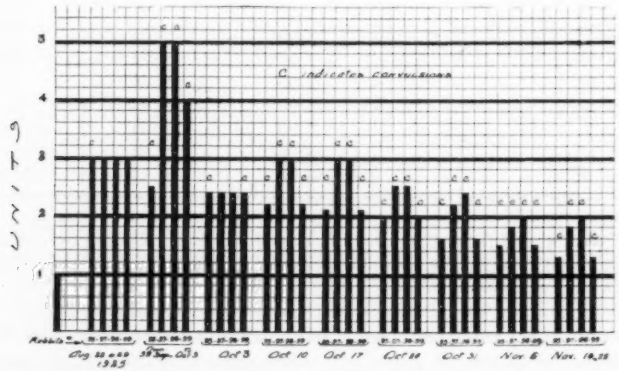

Fig. 4. Further data showing the decreasing resistance of rabbits with use. This is the phenomenon of "education." See text for a more complete description.

duction of convulsions. The blood sugar at the time of convulsions amounts to about 30 mgm. per 100 cc. We believe this indicates practically a complete disappearance of glucose from the blood. These findings confirm the recent observations of Benedict (4), and of Hiller, Linder and

Van Slyke (5) that approximately 25 per cent of the amount estimated as blood sugar is due to substances other than glucose. The method of Folin and Wu was used in these determinations. We use a hypo standard corresponding to a sugar content of 50 mgm. per 100 cc. in reading the low blood sugars.

Figure 3 shows the results obtained with an assay of another preparation of insulin. The strength of this was found to be 100 units per cubic

TABLE 2

Development of immunity to insulin

DATE	WEIGHT		UNITS INJECTED PER KILOGRAM
	Rabbit 91	Rabbit 92	
1925	*kgm.*	*kgm.*	
July 23	2.30	2.15	1.00
July 30	2.38	2.30	1.16
August 6	2.47	2.27	1.33
August 13	2.55	2.41	1.50
August 20	2.55	2.41	1.66
August 27	2.58	2.38	1.85
September 3	2.66	2.41	2.00
September 10	2.66	2.50	2.16
September 17	2.70	2.50	2.33
September 24	2.72	2.50	2.50
October 1	2.72	2.58	2.50
October 15	2.78	2.55	2.66
October 22	2.80	2.52	3.00
October 29	2.95	2.64	3.33
November 5	2.92	2.72	3.33
November 12	2.95	2.78	3.66
November 19	3.00	2.80	8.00
November 26	3.00	2.80	10.00
December 3	3.06	2.82	10.00
December 10	3.06	2.85	50.00*

* Total amount injected.

These rabbits were first given insulin March 26, 1925. They were "educated" and convulsed typically. The administration of sub-minimal convulsion doses resulted in a marked immunity.

centimeter. The parallelism of the two curves is apparent and shows that the standard rabbits react uniformly when different preparations are used.

The unit as determined by the above method may obviously have any value desired. As reported herein, the unit is equivalent to 1.0 clinical unit when checked against Lilly's U 80, 80390 758690. The unit as determined above has a lower value, because less resistant rabbits are used and because the intravenous method of administration is less effective than is the subcutaneous method.

In figure 4 are presented added data on the phenomenon of "education." We have stated that animals which have experienced insulin convulsions are less resistant than those which have never convulsed. The literature contains reports of practically the opposite nature in that immunity to insulin was said to have been established. The figure shows that of 4 rabbits which had never been used for any purpose nor had ever received insulin, only one convulsed when 3 units per kilo were given. The same result was obtained the following week. Two of these animals were then given 5 units per kilo for a period of three weeks. The following week a dose of 2.4 units per kilo was insufficient to produce convulsions but a dose of three units was successful the next week. After a period of twelve weeks, all of these rabbits convulsed on doses of between 1 and 2 units per kilo. We cannot emphasize too strongly that young, unused rabbits are more refractory to insulin than they are after convulsions have been repeatedly produced.

It is very likely that rabbits which are not kept on such a strict regimen as ours may develop immunity to insulin. Table 2 gives the records of two rabbits in which marked immunity developed. These animals were started on insulin March 26, 1925. They experienced convulsions weekly until July 23, 1925. They were then given insulin in doses just insufficient to produce convulsions and were not given a repeat dose on the same day. It has been possible to gradually increase the dose until on December 10, 1925, each of the rabbits received 50 units without having convulsions. These results indicate that immunity may develop when sub-minimal convulsion doses are given. We have never seen the development of immunity when rabbits are "educated" and then given convulsions every week.

The method of standardizing outlined herein is based upon the production of convulsions. These occur in our standard animals when the blood sugar reaches a level of about 30 mgm. per 100 cc. The time elapsing after the intravenous injections of insulin until the appearance of convulsions varies from 60 to 120 minutes. Each rabbit responds repeatedly within a narrow time limit. We believe that our conditions fulfill the possibility expressed by Macleod and Orr (2). They wrote: "Notwithstanding these criticisms, it is interesting that the assays by the two methods are usually not very far apart, and it is possible that future work may indicate how the physiological conditions of animals may be standardized so as to ensure a close correspondence between the blood-sugar level and the incidence of convulsions." The method has worked well in practice. Different insulin preparations, assayed as above, have been used clinically without experiencing difficulty in changing from one to another.

Believing that others may find our plan useful, we are giving a rather detailed description of the actual preparation and care of our rabbits.

The diet consists of good alfalfa hay with the addition of crushed barley after convulsion and on the following day. A 24-hour fast precedes the tests. Females about 3 months old are first used when they weigh from 1.25 to 1.50 kilos. Younger animals should not be used as they are likely not to thrive and are subject to ill health. Older rabbits are more resistant and more difficult to "educate." These young rabbits require about 5 units per kilo to produce convulsions. This dose is continued for two or three weeks and is then gradually decreased until a minimum is reached. This usually requires about 3 months. The rabbits are used every 7th day and are always given enough insulin to produce convulsions. Young animals should grow satisfactorily and mature ones should maintain their weights. Sickness of any kind renders a rabbit unfit for test purposes.

SUMMARY

A method of assaying insulin by the use of a few standard rabbits is described. The production of convulsions, which occur at a blood sugar level of about 30 mgm. per 100 cc., is the measure of potency. This measure can be used when the physiological conditions of the animals are suitably controlled. Further instances are given of the decreased resistance of rabbits to insulin after convulsions have been experienced.

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THE IDENTITY OF MUSCLE HEMOGLOBIN AND BLOOD HEMOGLOBIN[1]

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There have been interesting fluctuations in the theories or beliefs of physiologists as to the relationship between the fixed muscle hemoglobin and the circulating blood hemoglobin. About 100 years ago it was thought that the muscle was impregnated with hemoglobin and if not all the hemoglobin was in the capillaries, there was a labile exchange between the muscle fibres and the blood hemoglobin,—an identity of red pigment material in the contracting substance and in the circulation. About 50 or 60 years ago it was shown that perfusion of muscle did not wash out all the red color —that some of this coloring matter was firmly fixed in the striated fibres. The belief that these substances were distinct and different held sway for some time. Gradually, however, it was shown that these hemoglobin substances from muscle and blood had in common many characteristics. Hemin crystals can be formed from blood and muscle hemoglobin and these peculiar crystals are identical. The evidence as summed up by Günther (1) indicated that the pigment fraction of muscle and blood hemoglobin is identical but that differences are found in the *globin fractions*. This hypothetical difference in globin radicles we may say is difficult of proof one way or the other.

The idea that there is a labile exchange of hemoglobin between muscle and blood has been abandoned but there are some experimenters who claim a fairly rapid fluctuation of muscle hemoglobin in response to hemoglobin fluctuations in the blood. The preceding experiments show that anemia demands may change little or not at all the muscle hemoglobin levels. Such changes when they do occur are very slow adjustments, noted only after many weeks of severe anemia. The demands of the striated muscle for necessary hemoglobin are as urgent as the demands of the blood forming organs and so far as is now known neither can rob the other.

The muscle hemoglobin in dogs is quite necessary for life and its con-

[1] This work has been aided by a National Live Stock and Meat Board Fellowship of the National Research Council. We acknowledge with pleasure the friendly advice and assistance of Dr. E. B. Forbes and Dr. C. Robert Moulton.

centration appears to depend upon the amount of muscular exercise. Also the muscle hemoglobin seems to determine at least in part the reserve power of any given muscles. It is highly probable that the muscle hemoglobin is concerned with the rapid exchange of O_2 and CO_2 between the blood and contracting elements. It is a powerful buffer and may well be concerned with the reserve energy of striated muscle which is capable of such explosive liberation of energy. That this muscle hemoglobin heaps up in striated muscle during training or conditioning of the dog is significant in relation to some of the recent work by Hill (3).

It is very easy to extract this muscle hemoglobin from the muscles after they have been washed quite free from all circulating blood as described above (paper 1). Under these favorable conditions we have been able to make careful comparison between solutions of muscle hemoglobin and blood hemoglobin using an accurate spectrophotometer. The oxyhemoglobin curves from muscle or blood can scarcely be differentiated. The carbon monoxide hemoblobin curves show slight differences which are discussed in detail below but we may say that there is strong evidence that the two hemoglobin materials are identical. Such small differences as do appear seem to be best explained by slight ferment action going on in the muscle hemoglobin extract. It is possible of course that some pigment other than hemoglobin is extracted from the muscles and is in part responsible for very slight differences in the absorption curve.

There was considerable activity in the study of muscle hemoglobin between 1880 and 1890 with emphatic difference of opinion. The papers of MacMunn, (7) Levy (6) and Hoppe-Seyler (4) are adequately reviewed in a very recent paper by Keilin (5) and need not detain us here. It may be pointed out that these investigators utilized different methods of isolation and examination. Much work was done with living muscle examined in thin layers by a spectroscope. There are obvious difficulties in this procedure and strong probability of a number of pigment substances being concerned in the spectra described. Some of the materials examined certainly contained some circulating blood. Various reagents were used to split up the pigment before analysis and perhaps it is not surprising that the resultant confusion in the literature was impressive in its totality. In the present experiments an accurate specification of color is attained by employing a spectrophotometer and the absorption curves obtained for pure muscle extract are compared with blood hemoglobin solutions.

EXPERIMENTAL OBSERVATIONS. *Muscle extracts:* A detailed description of the preparation of these muscle extracts is given in the first paper of this series. These muscle hemoglobin solutions were quite clear and were examined in the spectrophotometer. In every case a part of the solution was saturated with illuminating gas and also examined spectrophotometrically. The blood solutions used for comparison were made by diluting

freshly drawn venous blood with a weak ammonia solution of the same strength as that used for muscle extraction. The CO compound was also prepared and subjected to spectrophotometric examination.

Spectrophotometer: Color measurements were made on a Bausch and Lomb spectrophotometer and from the data obtained, curves were plotted using wavelengths as abscissae and degree of absorption as ordinates. In all cases the thickness of layer of the solutions measured was 1 cm. The collinator slit of the spectroscope was varied in making the readings corresponding to points on the curves so as to secure approximately equal and minimal brightness (Fechtner's law). The width of slit was never over 0.1 mm. and at this maximum, the purity of spectrum was not materially impaired. The ocular slit was maintained at a constant width throughout which measured at the mercury green line 3.0 $\mu\mu$. The points plotted on the curves correspond to the mean wavelength of the spectrum which is included by the ocular slit. A detailed account of the instrument used and its calibration will be included in a forthcoming paper on "a spectrophotometric study of blood solutions."

Spectrophotometric measurements: The colored solution to be examined was placed in a cell 1.0 cm. thick and a similar cell was filled with the pure solvent. The cell which contained the solution was placed in one light beam of the photometer and the solvent in the other beam. Absorption measurements were made by rotating the analysing nicol. About 25 readings were made over the spectrum from 450 $\mu\mu$ to 630 $\mu\mu$. The cells were then reversed so that the solution was in the opposite beam and a similar set of readings taken. For each wavelength two different settings of the analysing nicol were obtained, designated by θ_1 and θ_2.

The photometer used was of the Martens type, therefore,

$$\text{Transmission} = \frac{\tan \theta_2}{\tan \theta_1} = \tan \theta_2 \cdot \cot \theta_1$$

and

$$\begin{aligned}\text{Density} &= -\log \text{transmission} \\ &= \log \tan \theta_1, + \log \cot \theta_2\end{aligned}$$

θ_1 is the scale reading of the analysing nicol for match when the sample is covering the half of the field which is extinguished for $\theta = 0°$, and will vary from 45° to 90°.

θ_2 is the scale reading of the analysing nicol for match when the sample is covering the half of the field which is extinguished for $\theta = 90°$; θ_2 will vary from 0° to 45°.

It would be superfluous to include as data all the angular readings and computations. The curves submitted herewith are considered as adequate specification of color measurement. The reason for using the term

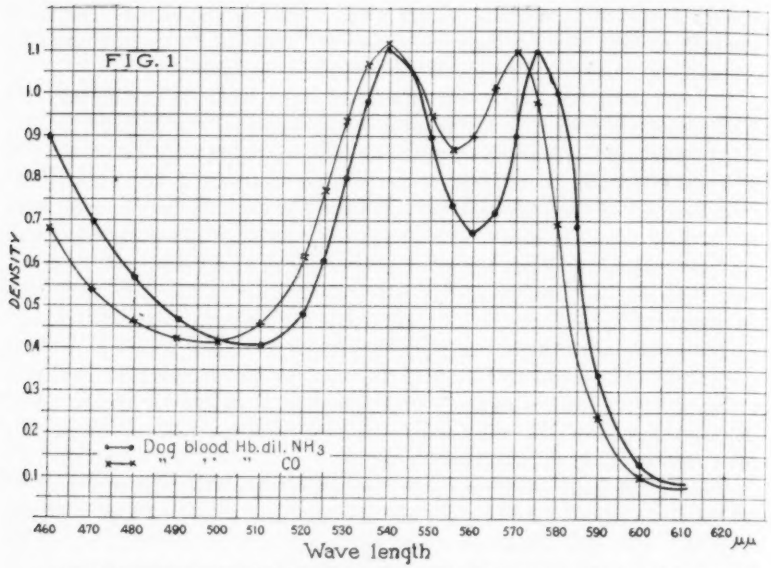


FIG. 1
DENSITY
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Dog blood Hb. dil. NH3
" " " CO
460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 μμ
Wave length

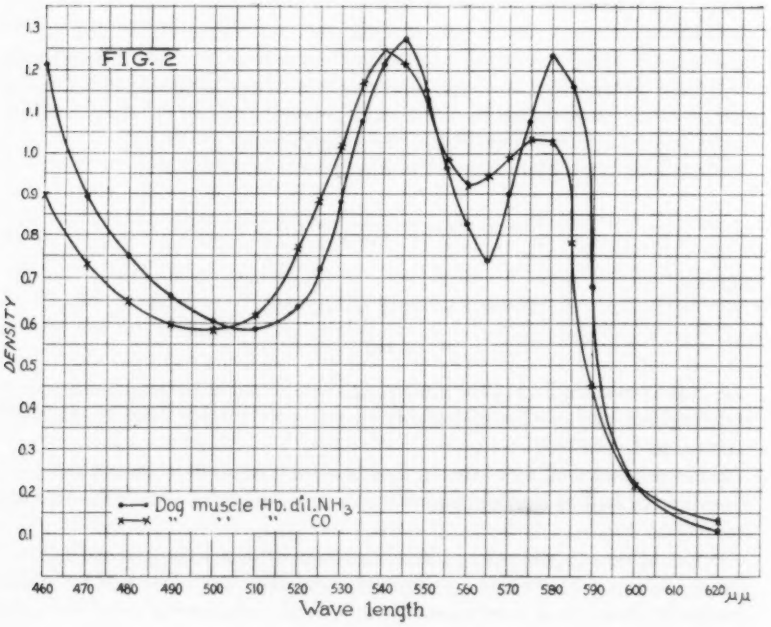


FIG. 2
DENSITY
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Dog muscle Hb. dil. NH3
" " " CO
460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 μμ
Wave length

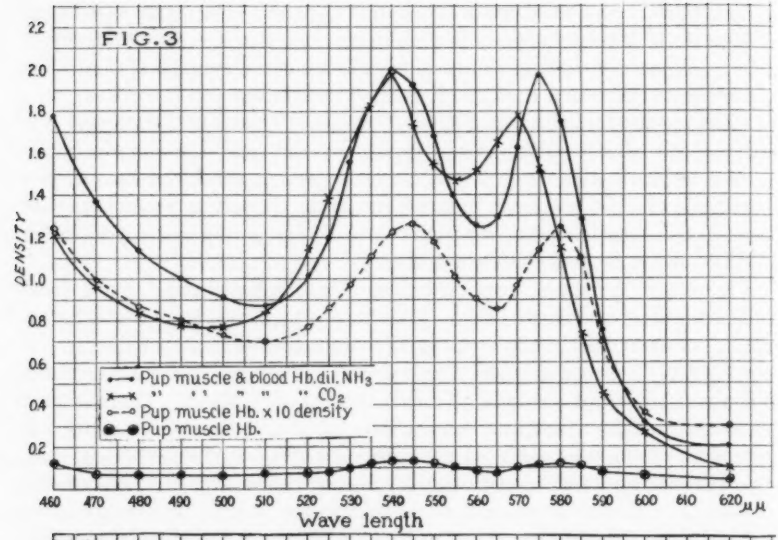
optical density as a measure of absorption instead of extinction coefficient is because of the confusion that sometimes occurs in the use of the latter term. When the thickness of a solution is 1.0 cm. the value of density is the same as extinction coefficient, defined by Bunsen and Roscoe.

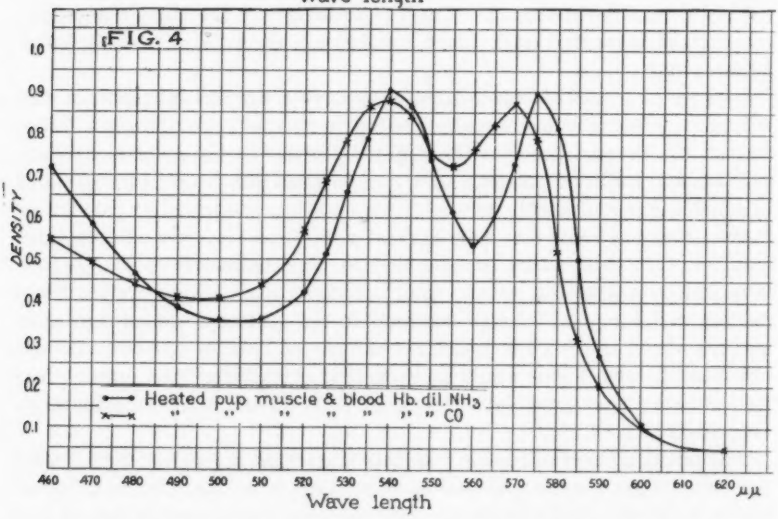
Spectrophotometric curves: Figure 1 shows the absorption curves for dog's blood diluted with 0.4 per cent ammonia, and the same solution after saturation with CO. These curves represent the blood hemoglobin standards to which the other data are referred. It may be noted here that no difference has been observed in the color of hemoglobin when dissolved in distilled water or in a weak alkali (2).

Figure 2 shows the absorption curves of a standard extract made from skeletal muscle of a normal dog. This muscle was freed from blood hemoglobin as described in the method of preparation of muscle extracts. A considerable number of adult dog muscle hemoglobin extracts have been examined spectrophotometrically and the curves plotted. There is such uniformity in the absorption curves that the single example in figure 2 is sufficient and may be regarded as typical of adult dog muscle hemoglobin. One curve in figure 2 corresponds very closely to the oxyhemoglobin curve of blood. There is, however, a slight shift in the absorption bands toward the red end of the spectrum. The CO derivative in the muscle hemoglobin extract compared with blood hemoglobin shows a noticeably paler yellow band.

It is well known that the solvent may have a marked influence on the color of a solution as in the case of iodine in alcohol or chloroform. Likewise in the present problem the slight difference in the color in a blood solution and a muscle extract might very well be a difference in the medium in which the pigment is dissolved. This question was tested by extracting in the usual way some very pale normal puppy muscle, then adding sufficient blood to this extract to make the color approximately as deep as that obtained by extracting muscle from an adult dog. This puppy muscle extract with blood added was examined in the spectrophotometer at once and the absorption curves, which are not shown here, were identical with the blood curves. This would indicate that extractives from puppy muscle would cause no appreciable change in the spectrum of blood under these experimental conditions.

The possible effect of muscle ferments was tested as follows: Practically colorless puppy muscle was minced and added to a 1 per cent solution of blood in 0.4 per cent ammonia. This mixture was placed in the ice box for two days. It was then filtered and figure 3 shows the corresponding absorption curves. Here, there is a noticeable change in the CO derivative which approaches the findings in figure 2. To show the relative amount of muscle hemoglobin and any other hypothetical pigment extractives in normal pup muscle we include in figure 3 the lowest curve. It is





obvious that this muscle extract alone would scarcely modify the combined hemoglobin curve. The color of the puppy muscle extract without added blood was so pale that absorption measurements were made with a 10 cm. cell or ten times the thickness that was used in all other experiments. The actual values are shown by the dotted curve. The lowest curve in figure 3 represents these values reduced to the scale of the other curves.

In order to check the ferment action as a causative agent a similar experiment was done except that the puppy muscle was previously heated to 60°C. for one hour. This mixture was kept four days in the ice box and then examined. Figure 4 shows the resulting curves are in all respects similar to those obtained from normal blood hemoglobin.

DISCUSSION. The spectrophotometric curves here shown represent an accurate specification of the color of muscle extracts. Compared to similar curves made from blood, a remarkable degree of similarity is apparent. It seems highly improbable that the pigments of muscle and blood are essentially different in chemical constitution when their colors are so nearly alike.

The slight differences noted in the muscle and blood curves may be explained by a slight decomposition of the pigment due to ferment activity on the part of the muscle during the process of extraction. Examination of the curves of figures 3 and 4 would suggest this. Again, it is not unlikely that there are other colored substances beside hemoglobin which may be obtained in some muscle extracts which would alter the character of a pure hemoglobin absorption curve. In this connection we are aware of the work of Keilin (5) on a pigment which he calls "cytochrome," which he has found in practically all tissues. Keilin's spectroscopic description of the pigment is too meagre for us to postulate what effect its presence would have in altering a hemoglobin absorption curve. Whether "cytochrome" is extracted from the dog muscle by this method we cannot say. The fact that extracts of puppy muscle plus blood examined spectrophotometrically at once, gave curves identical with blood hemoglobin, would indicate at most an insignificant amount of "extraneous" pigment compared with the amount of red pigment in muscles of the adult dog.

SUMMARY

Solutions of *muscle hemoglobin* free from blood hemoglobin examined by the spectrophotometer give curves very similar to the curves obtained from *blood hemoglobin*.

Slight differences in these curves may be explained by a slight amount of ferment action which modifies the muscle hemoglobin during the process of extraction.

One may choose to postulate an unknown pigment substance in these muscle extracts to explain in part these slight differences. We believe that the evidence in favor of this explanation is very weak.

This work is in harmony with that of investigators who tend more and more to look upon muscle hemoglobin and blood hemoglobin as almost indistinguishable.

Even if we assume that muscle hemoglobin and blood hemoglobin are chemically identical we must go further and ascertain how similar or

dissimilar may be the story of construction and destruction within the body of these two important hemoglobins. We hope to report work of this nature in the near future.

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THE HEMOGLOBIN OF STRIATED MUSCLE[1]

I. Variations Due to Age and Exercise

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In contrast to the enormous amount of work dealing with the hemoglobin of the blood one finds only occasional papers concerned with the hemoglobin of striated muscle. Yet it is probable that the one is fully as important in body economy as the other. Even the mass of muscle hemoglobin in the dog is considerable and may vary in amount from 15 to 50 per cent of the total blood hemoglobin. That the wastage and repair of this muscle hemoglobin are of considerable importance as regards the body pigment metabolism cannot be denied. Any investigator who studies certain end products of hemoglobin disintegration (bile pigments and urobilin) and draws deductions without due consideration of the muscle hemoglobin is indeed building a house upon the sands.

One of the favorite indoor sports of students of the body pigments is the determination of the life cycle of the red blood cell from analysis of one or another pigment—usually stercobilin. The most recent contributors to this perennial debate are Lichtenstein and Terwen (7) who claim great accuracy for the method used to measure urobilin. This accuracy is praiseworthy but it contributes nothing to our knowledge of the life cycle of red cells which they establish as 110 to 140 days. There are at least two unknown factors which could seriously disturb these calculations. First, the muscle hemoglobin certainly contributes end products of the nature studied. Second, hemoglobin, freed in the circulation *may* be changed to bile pigment and urobilin or it may be carefully reserved for new hemoglobin construction. Under favorable conditions as much as 50 to 75 per cent of injected hemoglobin may be conserved and consequently upset any calculation based on elimination of all hemoglobin wasted from red cells (9).

Hemoglobin regeneration in anemic dogs has been studied in this laboratory for several years (9). As a part of this experimental program it

[1] This work has been aided by a National Live Stock and Meat Board Fellowship of the National Research Council. We acknowledge with pleasure the friendly advice and assistance of Dr. E. B. Forbes and Dr. C. Robert Moulton.

seemed necessary to study with considerable care the hemoglobin content of various muscles in dogs during various life periods. Some of these experimental data are given in tables below which indicate the variations to be observed in age periods, in muscle groups and in disease conditions. A comprehensive review of the older literature is not needed as a recent paper by Günther (3) gives this information. There are only a very few papers since 1900 which deal with muscle hemoglobin and the methods used are grossly inaccurate as a rule. Most of the work concerns other animals than dogs so that from our point of view there are no papers of any significance dealing with quantitative values of muscle hemoglobin.

Günther (3) studied chiefly muscle from guinea pigs and rabbits washed free from blood by a cannula in the aorta. He used a watery extract of the muscle. This method is inaccurate and gives solutions very hard to match with blood. He compared his solutions with an arbitrary blood standard but gives no figures as to the exact hemoglobin strength of such solutions. He studied also calf hearts. He reports (4) observations on hemoglobinuria in man associated with myositis. Feigl (2) reports an interesting hemoglobinuria in troops after long marches. This probably has an interesting relationship to the observation of Brown, McMaster and Rous (1) that there is an increase in bile pigment after vigorous exercise in the dog. Brown, McMaster and Rous explained their findings on the basis of red cell disintegration but we believe the evidence goes to show that muscle hemoglobin disintegration is the more important factor. We shall have more to say on this subject in the near future.

Lehman (6) gives some data on the content of muscle hemoglobin in meat. He uses fresh meat from the slaughter house ground with sand and extracted with water. This method gives a turbid solution impossible to compare accurately with a blood standard. He claims that washing out of the blood is not necessary. This sounds improbable and is shown to introduce gross errors in experiments given below (table 6). His standard is diluted blood. He studied a large series of animals including cattle, game, birds, rabbits and man but we consider his values quite inaccurate. Lankaster (5) made some interesting qualitative observations on molluscs. He studied some forms which showed hemoglobin in the muscles and no hemoglobin in the circulation. Other forms show no hemoglobin in muscles and true hemoglobin in the circulation. These were important data to support the then recent claim that muscle hemoglobin was quite separate from circulating hemoglobin. This may have given support to the many who claimed at various times that muscle hemoglobin is quite different chemically as well as physiologically from blood hemoglobin.

METHOD.[2] Much time was spent in devising an accurate method capa-

[2] The writer is greatly indebted for valued technical assistance to Priscilla A. Hutchens, Monica Walsh, Bessie L. Bodine and Anna Hamman.

ble of giving hemoglobin values for muscles which could be duplicated independently by various workers. In all the experiments given below at least two sets of readings were made from separate specimens of muscle tissue. Usually these readings are averaged into a single figure but some experiments are given (table 5) to show the normal variations due to personal and method shortcomings.

The animal is freed from blood by a technique used in other experiments to wash out plasma proteins. Under ether anesthesia cannulas are tied into the carotid and jugular vessels. A Ringer's solution with 5 per cent glucose at body temperature is injected by gravity into the jugular vein at the same speed as blood is drawn from the carotid. By slow and careful simultaneous bleeding and transfusion one can reduce the red cell hematocrit from 50 per cent (or normal) to very low values of ½ to 3 per cent. When the heart stops the thorax and abdomen are opened immediately, the heart and viscera removed and legs and trunk massaged to facilitate drainage of the little blood remaining in the tissues into the body cavity. After this procedure the various muscles are dissected free with no loss of time and the samples are rinsed and massaged in normal saline. The samples are then dried carefully with a towel and the sample taken for extraction. Care is taken not to include fat and fascia in this sample. A muscle sample obtained in this way is free from blood hemoglobin and careful search in frozen or fixed sections will not reveal any red cells in the capillaries.

In our experiments this simultaneous bleeding-perfusion method is infinitely superior to simple gravity perfusion with normal saline. Our method causes no tissue edema which is the rule with gravity perfusion and edema introduces a serious error in quantitative muscle hemoglobin measurement. The washing out of the tissues and viscera by the heart pulsations with a constant decrease in the circulating hemoglobin is most satisfactory.

The muscle may be ground in a small meat grinder or cut up into a fine granular mass with scissors or a meat chopper. Faithful attention to the fine division of the muscle tissue is quite necessary for accurate work. In our hands grinding with sand is undesirable as it gives a turbid solution which cannot be cleared nor read accurately against the standard hemoglobin solution. Of the finely cut muscle tissue are weighed out exactly 10 grams and made up to 50 cc. with an 0.4 per cent ammonia solution. This mixed muscle extract is put in a bottle in the ice box (2° to 4°C.) over night and after 20 hours prepared for colorimetric reading. The muscle extract is filtered through cloth and squeezed thoroughly giving a somewhat turbid red solution. This is now filtered through double filter paper giving a clear red solution which is treated with CO to saturation and read against a 1 per cent CO hemoglobin blood standard made

and standardized after the oxygen capacity method of Van Slyke. A 10 cc. portion of the red filtrate is treated with 4 drops of concentrated HCl, heated to 56°C. in a water bath for one half hour, cooled and read against a standard acid hematin solution. The CO fraction and acid hematin fraction give values which usually agree very closely. At times there may be difficulty with the CO solution which does not match the standard exactly. This is in part due, we believe, to muscle autolysis as discussed elsewhere but when the CO method and the acid hematin method agree we feel confident that our determinations are accurate.

We used other solutions to extract the muscle with less complete extraction or color impairment. This was true of distilled water used by many of the older investigators and other alkali solutions of varying concentration.

TABLE 1

Muscle hemoglobin in milligrams per 100 grams; pups less than 1 month of age

	DOG NUMBER					
	4	5	6	7	15	Average
Diaphragm		276	330	452		352
Heart	352	276	260	332	191	282
Fore leg	137	174	150	130	126	143
Hind leg	153	188	110	115	65	122
Blood Hb. in per cent	50	55	43	41		47
Age in days	12	14	15	15	28	
Weight in kgm	0.31	0.42	0.35	0.31	4.4	

Pups 4, 5, 6 and 7 bled but not perfused.

Pup 15 killed the day before, preserved in ice box and dissected about 20 hours later.

In the great majority of the experiments tabulated below only one figure is given for each muscle but this represents two sets of determinations by two workers making independent readings. Each worker measured the muscle extract by the CO method as well as by the acid hematin method. The names of the muscles used in the tables indicate easily isolated muscles which can be dissected free from fat and fascia with little difficulty. The word "spinae" indicates a liberal sample taken from the erector spinae group between the ileum and the lower ribs. These experiments convinced us that accurate quantitative determination of muscle hemoglobin is possible using the methods described.

EXPERIMENTAL OBSERVATIONS. A good many experiments not here recorded were incomplete in one detail or another. In some the CO method did not give colors which could be compared accurately with the standard and in such instances we place main reliance upon the acid hematin figures. All these data, however, support these published experiments

which are good examples of a large series. There are interesting differences in hemoglobin content of the striated muscles which appear to relate to age, muscular strength or functional demands.

It is a familiar observation that young animals have pale muscles as compared with adults of the same species—for example, veal and beef contrast in color; most of this color is in the muscle fibres rather than in the capillaries. The striated muscles of the legs and trunk in young pups are very pale as compared with the deep red muscles of the adult dog and we see expected differences in the muscle hemoglobin figures ranging from 100 to 150 in pups to 700 to 1000 in adults. Table 1 shows somewhat high

TABLE 2

Muscle hemoglobin in milligrams per 100 grams; pups 6 to 10 weeks of age

	DOG NUMBER			
	16	14	17	Average
Diaphragm	136	175		155
Heart	119	146	119	128
Rectus	51	135	70	85
Pectoral	89	105	85	93
Triceps	93	124	86	101
Flexors	105	132		118
Vastus	95	147	108	117
Adductors	132	167	133	143
Gastrocnemius		149		149
Spinae	185	164	152	167
Average legs and back	125	147	119	132
Blood Hb. in per cent	63	73	74	70
Weight in kgm.	0.79	4.18	1.10	2.02
Age in weeks	6	10	7	

Simultaneous bleeding and perfusion to wash out all circulating hemoglobin.

values for heart, diaphragm and skeletal muscles as compared with older pups (tables 2 and 3). We believe this is due to the lack of perfusion and the presence of residual blood in the capillaries—see also table 4 for further evidence.

Tables 2 and 3 show different pups ranging from 6 to 11 weeks of age and from 0.79 to 4.81 kgm. in weight. In general these perfusion experiments indicate the true muscle values for young pups. We observe that the *heart* averages about 120 mgm. hemoglobin per 100 grams which is proportionately higher than we find in adults where heart values are far below the striated skeletal muscle. Also the *diaphragm* averages about 125 to 150 mgm. hemoglobin which is higher proportionally than in adults. It is probable that the work of the heart and diaphragm in young pups calls for earlier development of muscular strength and consequent increase in

muscle hemoglobin. As in the older dogs we see that the flat muscles of the thorax and abdomen are poorer in hemoglobin than the leg muscles—

TABLE 3

Muscle hemoglobin in milligrams per 100 grams; pups 9 weeks of age

	DOG NUMBER			
	11	12	13	Average
Diaphragm	85	103	155	114
Heart	101	125	124	117
Rectus	50	56	52	53
Pectoral	52	65	60	59
Triceps	55	67	61	61
Flexor	83	78	82	81
Vastus	55	93	75	74
Gastrocnemius	85	138	96	106
Adductor	58	83	67	69
Spinae	61	88	94	81
Average legs and back	66	91	79	79
Blood Hb. in per cent	47	52	50	
Weight in kgm	2.0	2.3	2.4	

Simultaneous bleeding and perfusion to wash out all circulating hemoglobin.

TABLE 4

Muscle hemoglobin in milligrams per 100 grams; pups of 4 months of age

	DOG NUMBER			
	88	24	10	9
Diaphragm	199		252	678
Heart	193	205	279	514
Rectus	140		139	338
Pectoral	151		205	390
Right triceps	174	205	235	408
Left triceps	177	198	232	402
Flexors	212	219	217	468
Right vastus	171	195	240	322
Left vastus	152	197	242	305
Right adductor	168	225	206	380
Left adductor	166	202	208	394
Gastrocnemius	172	228	215	363
Spinae	187	240	248	400
Average legs and back	175	212	227	382
Blood Hb. in per cent	82	93	53	52
Weight in kgm	6.37	4.59	3.75	4.54
	Perfused	Perfused	Bled	Not bled

this applies not only to the rectus and pectoral but to the platysma and various obliques of the abdomen. It is probable that the presence of more fascia in these muscles will explain a portion of this difference.

The pups given in table 3 were from a single litter and present unusual uniformity. The heart and diaphragm show higher values than any of the skeletal muscles which are somewhat below the usual average for pups of this age.

Table 4 shows pups of 4 months of age that are becoming increasingly active. They have been weaned and show some increase in blood hemoglobin as well as muscle hemoglobin. The leg muscles begin to equal the heart and diaphragm in hemoglobin content. The influence of bleeding,

TABLE 5

Muscle hemoglobin in milligrams per 100 grams; complete perfusion

	DOG 24-98		DOG M-96	
	CO	Acid	CO	Acid
Diaphragm	377	422	253	251
Right ventricle	352	362		
Left ventricle	337	335	264	245
Right rectus	312	328	253	251
Left rectus	300	300	210	214
Right pectoral	357	351	212	208
Left pectoral	351	351	208	215
Right triceps	570	580	245	262
Left triceps	545	550	242	297
Right flexor	520	520	265	314
Left flexor	565	570	269	314
Right vastus	493	550	228	264
Left vastus	406	422	270	254
Right gastrocnemius	479	472	262	293
Left gastrocnemius	472	470	252	
Right adductor	565	545	262	275
Left adductor	580	590	285	297
Right spinae	490	510	280	297
Left spinae	510	555	272	300
Average legs and back	516	528	261	288
Blood Hb. in per cent	120		112	
Weight in kgm	14.5		8.37	
Age in months	8		7	

lack of any bleeding and perfusion comes out very clearly in table 4. It is obvious that bleeding helps considerably (compare no. 10 and no. 9) but perfusion is needful to give accurate values for muscle hemoglobin (compare nos. 88 and 24—table 4). It is obvious that with muscles of low hemoglobin content only a very little blood in the capillaries is needed to modify the true values. These pups average 2 or 3 times as much muscle hemoglobin per 100 grams as the pups in table 3. These larger pups however, show a less marked increase in heart and diaphragm muscle hemoglobin over the pups in table 3.

Table 5 gives two experiments on dogs of very different character. The various readings of the muscles on the two sides are given as a fair example of the variations encountered in routine experiments and as a control for subsequent experiments. Dog 24-98 (table 5) was a young male raised in our kennels, just matured, perfectly normal and active. His leg muscles are showing rapidly increasing values in muscle hemoglobin which are much above the diaphragm and heart values. The recti and pectorals show the

TABLE 6

Muscle hemoglobin in milligrams per 100 grams; operation and perfusion

	DOG NUMBER			
	M-80	M-85	M-77	Average
Diaphragm	405	353	365	374
Left ventricle	248	303	325	292
Right rectus	305	331	365	334
Left rectus	307	298	323	309
Right pectoral	300	343	348	330
Left pectoral	330	345	333	336
Right triceps—operation	494	455	653	534
Right triceps—not perfused	398	330	373	367
Left triceps	365	363	430	386
Right flexor—operation	614	500	678	577
Right flexor—not perfused	480	454	430	455
Left flexor	445	405	430	426
Right vastus—operation	385	375	500	416
Right vastus—not perfused	385	350	430	388
Left vastus	362	345	401	369
Right gastrocnemius—operation	405	430	550	462
Right gastrocnemius—not perfused	445		405	425
Left gastrocnemius	395	385	385	388
Right adductor	365	388	380	378
Left adductor	365	388	387	380
Right spinae	365	398	356	373
Left spinae	365	380	415	387
Blood Hb. in per cent	108	86	101	
Weight in kgm	12.3	15.4	14.1	
Age in months	9	9	8	

lower values common in adults. This dog shows higher values for leg muscles and hemoglobin than other outside pups of about the same age (table 6) but we believe this is due at least in part to the more favorable diet in our kennels. We expect to furnish experimental data relating to muscle hemoglobin and diet in the near future.

Dog M-96 was raised outside the kennels and had been sick with distemper, refusing food for 7 days. He was weak and sick at the time of the perfusion. His blood hemoglobin level is not very low, but the muscle hemo-

globin is decidedly subnormal. His diaphragm and heart show values about equal to the leg muscles.

Table 6 gives experiments performed in an attempt to remove control samples of muscles. These experiments illustrate the need of perfusion and massage of muscles to insure complete removal of circulating hemoglobin. A successful method was finally evolved for the removal of control muscle samples (table 21) but these experiments (table 6) are of value in this place to supply data on the necessity of complete removal of circulating hemoglobin.

TABLE 7

Muscle hemoglobin in milligrams per 100 grams; strong healthy adult dogs

	DOG NUMBER					
	67-M	86-M	72-M	71-M	75-M	Average
Diaphragm		666	697	805	726	723
Left ventricle	770	404	509	320	393	479
Right rectus	1055	693	697	644	748	767
Left rectus	1091	670	603	670	728	752
Right pectoral	875	567	497	716	666	664
Left pectoral	870	575	543	635	651	665
Right triceps	983					
Left triceps	955	751	662	753	846	793
Right flexor	1017					
Left flexor	1067	780	686	865	806	841
Right vastus	1149					
Left vastus	1109	760	735	719	786	822
Right gastrocnemius	1119					
Left gastrocnemius	1122	742	753	767	706	818
Right adductor	1031	713	746	870	805	833
Left adductor	1030	723	680	657	806	779
Right spinae	1086	753	747	759	806	830
Left spinae	950	762	747	765	810	807
Average back and legs	1051	748	719	769	796	816
Blood Hb. in per cent	140	107	127	143	155	
Weight in kgm.	14.8	11.7	17.7	14.7	25.4	

In each of these three experiments (table 6) the same technique was employed. Under ether anesthesia the femoral and brachial arteries were ligated. The triceps, flexor group, vastus femoris and gastrocnemius were exposed individually and a sizeable piece cut away as rapidly as possible. This removed piece was mopped in normal saline, dried and extracted as usual. There was considerable escape of blood however, and the analyses show high values as compared with the other side, indicating considerable residual blood. After these operative procedures the perfusion-bleeding method was carried through as usual. The perfusion of the right fore and hind legs was obviously incomplete and effected by means of collateral vessels (ligation of femoral and brachial arteries).

The perfused leg and trunk muscles average about 390 mgm. muscle hemoglobin—the diaphragm slightly less. The heart contains 290 mgm. which is close to the normal adult values.

Table 7 shows the muscle values of 5 strong adult dogs. The first dog is unusual (67-M) but the others show average figures. We see that the leg and back muscles average 800 mgm. muscle hemoglobin. The diaphragm and flat muscles still lower and average between 400 and 500

TABLE 8

Muscle hemoglobin in milligrams per 100 grams; healthy adult dogs

	DOG NUMBER			
	84-*M*	78-*M*	81-*M*	AVERAGE
Diaphragm	387	682	470	513
Left ventricle	296	358	430	361
Right rectus	335	490	515	446
Left rectus	306	572	546	475
Right pectoral	365	443	455	421
Left pectoral	350	456	472	426
Right triceps—operation	509	945	465	639
Right triceps—not perfused	375	638	560	524
Left triceps	380	640	565	528
Right flexor—operation	374	987	750	704
Right flexor—not perfused	558	719	612	629
Left flexor	405	760	610	592
Right vastus—operation	363	677	513	518
Right vastus—not perfused	372	656	582	536
Left vastus	340	655	559	518
Right gastrocnemius—operation	394	789	630	604
Right gastrocnemius—not perfused	506	745	635	619
Left gastrocnemius	365	731	609	568
Right gluteal	375	650	585	536
Left gluteal	365	649	585	533
Right spinae	365	647	582	531
Left spinae	375	657	558	530
Blood hemoglobin in per cent	85	106	93	
Weight in kgm	4.54	11.4	22.7	
Average back and legs	371	673	581	542

mgm. muscle hemglobin. The first dog (67-M) deserves special mention because of the unusually high values. This was a hunting dog in perfect condition with no fat and gave every evidence of much vigorous exercise. This explains, we believe, the high values of the legs and back muscles (1050 mgm. muscle hemoglobin) and the heart muscles value of 770. Contrast with these figures the low values of an adult house dog 84-M, table 8. This poodle was certainly limited in exercise and shows leg and back muscles of 371 and heart 296.

Table 8 shows wide variation in values which are probably explained in part by exercise and diet. The first dog 84-M, a small poodle, was surely limited as to bodily exercise. It may have suffered from faulty diet as indicated by the low blood hemoglobin values but it is certain that this degree of anemia could not explain the low values of muscle hemoglobin (compare table 22). It is possible that faulty diet may have contributed directly to the low muscle hemoglobin values. Dog 78-M was pregnant about one month and these figures are within normal limits. Dog 81-M was normal in all respects but we cannot say as to muscular exercise.

TABLE 9

Muscle hemoglobin in milligrams per 100 grams; old dogs

	DOG NUMBER			
	82-M	87-M	95-M	Average
Diaphragm	583	667	512	587
Left ventricle	695	449	301	481
Right rectus	513	702	485	566
Left rectus	510	711	480	567
Right pectoral	451	625	432	502
Left pectoral	462	606	490	516
Right triceps	657		514	585
Left triceps	657	844	650	717
Right flexor	755		647	701
Left flexor	715	850	697	754
Right vastus	667		585	626
Left vastus	682	726	563	657
Right gastrocnemius	647		663	655
Left gastrocnemius	634	797	620	684
Right adductor	557	857	476	630
Left adductor	592	811	497	633
Right spinae	562	807		685
Left spinae	559	798		678
Blood hemoglobin in per cent	107	119	124	
Weight in kgm	15.2	22.8	8.91	
Average back and legs	640	811	591	680

This table like table 6 gives the results of operative removal of muscle samples—incomplete perfusion of the operated side and the normal perfused control side. The various steps were explained under table 6 and need not be repeated.

Table 9 shows some interesting abnormalities which influence the muscle hemoglobin content. The first dog 82-M was a very old bull dog. Emphysema and anthracosis were marked. Perfusion was not as complete as usual and the figures may be a little higher on that account. There was marked chronic nephritis which together with emphysema was

responsible for some cardiac hypertrophy and notable increase in heart muscle hemoglobin. Evidently great increase in heart work calls for more heart muscle hemoglobin as does increased exercise call for more skeletal muscle hemoglobin.

Dog 87-M (table 9), an old collie, shows high values for an old dog probably explained at least in part by an active outdoor life and exercise. This dog showed no abnormalities and there was little anthracosis, pointing to a country residence.

Dog 95-M (table 9) an old brindle bull female, 12 years of age. This dog was a house dog whose entire life was spent under observation of the

TABLE 10

Striated muscle per cent of body weight

DOG	BODY WEIGHT	MUSCLE WEIGHT	MUSCLE PER CENT
	kgm.	*kgm.*	
Strong, fat, long haired, adult male	22.59	5.660	25.05
Strong, lean, short haired, adult male	24.09	9.279	38.66
Young fat female	7.72	2.105	28.57
Average, adult, short haired male	11.74	4.191	35.91

TABLE 11

Muscle hemoglobin in milligrams per 100 grams; maximal and minimal values for dogs during age periods

	AGE					
	1 month	2 months	4 months	7–9 months	1–5 years	10–12 years
Leg and back muscles	100–150	80–140	170–200	280–500	350–1050	600–800
Heart	200–350	100–140	190–200	260–350	300–770	300–700
Diaphragm	250–400	80–170	200–250	250–400	390–800	500–650
Blood Hb. in per cent	40–55	50–70	50–90	90–120	85–155	100–125

owner. She did not get vigorous exercise. The dog was killed because of a breast tumor, which, however, had not as yet impaired the general condition. The heart and leg muscles show low normal values.

We wished to know the general percentage of body weight made up by the striated muscles in the dogs used in our work. For this reason we dissected 4 dogs of quite different type as shown in table 10. We believe these figures are accurate to within 1 to 2 per cent and they show physiological variations from 25 to 40 per cent depending upon muscular development, fat deposit, type of coat and skeleton. We feel that the average adult dog in good condition will average one-third of the body weight as muscle tissue. It is probable that part grown pups would run below 20 per cent of body weight as muscle tissue.

Table 11 shows the general values for the various muscles during various age periods. It is easily seen that the heart muscle and diaphragm start above the leg muscles in hemoglobin content. It is obvious that the energy demands upon the heart and diaphragm in the first month of life are much greater than upon the leg muscles. At about the 4th month of life the leg muscles equal the heart and diaphragm values. After this age the diaphragm and leg muscles are consistently above the heart muscle in muscle hemoglobin content.

Discussion. We may illustrate the great variations which may be encountered in a study of the total muscle hemoglobin by a few examples. We may have an average healthy, active, adult dog of 20 kilos. For the sake of simplicity we will assume a normal 9 per cent blood volume of 1800 cc.—further, a hemoglobin percentage of 130 per cent using 13.8 grams per 100 cc. as standard. This dog will have a circulating hemoglobin mass of $18 \times 13.8 \times 1.30 = 323$ grams. This same average dog may have 35 per cent body weight as striated muscle of a content of 700 mgm. per 100 gram which means $20{,}000 \times 0.35 \times 0.007 = 49$ grams muscle hemoglobin. As a second example we may use a rather fat dog of quiet type. This dog of 20 kilos weight may have the same blood volume but a hemoglobin level of only 100 or 248 grams of hemoglobin in circulation. The muscle tissue may make up only 30 per cent of the body weight and contain only 600 mgm. muscle hemoglobin per 100 grams which means 36 grams total muscle hemoglobin. In both instances the total muscle hemoglobin makes up about 15 per cent of the total blood hemoglobin.

By contrast let us assume a very vigorous lean hunting dog of 20 kilos weight. This dog may have been made anemic by experimental bleeding to a level of 40 per cent hemoglobin. Such anemic dogs have a blood volume of 8 per cent body weight. This dog will have a circulating hemoglobin of 88 grams. This dog may have a muscle weight of 40 per cent total body weight and a muscle hemoglobin content of 900 grams, which means a total muscle hemoglobin of 72 grams or 82 per cent of the total blood hemoglobin. We might cite another example of a young pup with pale muscles and low muscle weight which would show a muscle hemoglobin of less than 10 per cent of the total blood hemoglobin.

It may be accepted without further comment that the total muscle hemoglobin may vary greatly in relation to the total circulating hemoglobin. But great as is this variation how much do we know about the rapidity of disintegration of muscle hemoglobin? Assuredly it must be more rapidly broken down during vigorous exercise. Is it more rapidly used up than the hemoglobin of red cells in the circulation? May its end products be conserved and recast into new hemoglobin? Is there any interchange or interrelationship between circulating hemoglobin and muscle hemoglobin? All these things have a bearing upon the output of body

pigments and these questions and many others must be answered before we can understand the whole story of hemoglobin construction and disintegration. Probably some time will elapse before these problems are solved.

We have observed that the heart muscle in adult dogs has a distinctly lower muscle hemoglobin content than the powerful leg muscles. We may choose to explain this on the basis of the continuous and more nearly uniform cardiac activity of the normal dog as contrasted with the explosive and transient activity of the leg muscles. It is of interest in this connection to recall that the feeding of cardiac muscle to anemic dogs results in the production of more grams of blood hemoglobin than does the feeding of the highly pigmented skeletal muscle of the ox (8). We suggest that this indicates the storage within the heart muscle of parent pigment substances which may be a reserve against the constant wear and tear and replacement of the cardiac muscle hemoglobin.

SUMMARY

The *leg and back muscles* show a great range in their muscle hemoglobin content which appears to depend upon exercise and to determine largely the latent muscular power. Pups of 2 to 3 months of age may average 100 mgm. muscle hemoglobin per 100 grams. At four months there may be a rise of 200 mgm. and at 6 or 7 months 200 to 300 mgm. At puberty (8–9 months) we are apt to see a sharp jump in the curve to 400 or 500 mgm. per 100 grams muscle tissue. Adult dogs may vary all the way from 400 mgm. in a quiet house dog to 1000 mgm. in an active trained hunting dog.

The *diaphragm* shows high values during the first and second month of life—above the leg muscles. Not until near puberty does the diaphragm fall a little behind the leg muscles in hemoglobin content.

The *flat muscles* of the trunk—recti and pectorals—are uniformly below the leg muscles in hemoglobin content.

The *heart* is much more uniform in its muscle hemoglobin content just as is its work more steady and uniform than the work of the skeletal muscles. In the first few months of life the hemoglobin content of the heart muscle may be 100 to 200 mgm. per 100 grams. It may rise slowly to 300 at puberty and may never go much above this in a quiet house dog. Heart muscle values may average 300 to 400 for common adult dogs. Unusual exercise may push these values up to 700 mgm. of muscle hemoglobin per 100 grams. We may see the same high values with cardiac hypertrophy due to chronic nephritis and emphysema.

A quantitative method for the estimation of muscle hemoglobin is described.

Normal dogs may have 25 to 40 per cent of their entire body weight represented as striated muscle tissue.

Representing total circulating hemoglobin of the dog as 100 grams we may find the total muscle hemoglobin amounting to 10 to 80 grams in widely different conditions. It is obvious that muscle hemoglobin is of importance whether one studies the end products of hemoglobin disintegration or the parent substances suitable for construction into mature hemoglobin. Muscle hemoglobin must be considered in any study of body pigment metabolism.

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THE HEMOGLOBIN OF STRIATED MUSCLE[1]

II. Variations Due to Anemia and Paralysis

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The experiments given below indicate that prolonged anemia may lower somewhat the hemoglobin content of dogs' skeletal muscle but this level will scarcely go below values of 500 mgm. hemoglobin for 100 grams muscle. Muscle paralysis due to nerve section will cause a great lowering of the level of muscle hemoglobin. This fact supports the evidence given in the preceding paper that vigorous exercise in the normal dog is largely responsible for the high muscle hemoglobin values recorded.

Three very brief papers by Camus and Pagniez (1), (2), (3) deal with these conditions in dogs but these publications give no experimental data which would enable one to understand why some of their conclusions are in accord with our experiments and others are not. They mention a few dogs, usually one for each type of experiment but give no absolute values for either blood or muscle hemoglobin. Their method in our hands is inaccurate. They state that anemia causes reduction in muscle hemoglobin of the skeletal muscle as well as the heart but give no data on controls. Their anemia periods are short or not specified—nor is the degree of anemia established. We have been unable to ascertain wherein their experiments are faulty and can only conclude that they have neither proved nor disproved the various statements published.

Experimental observation. The two experiments outlined in table 21 show that there may be moderate lowering of the muscle hemoglobin level due to severe and prolonged anemia lasting 11 to 12 weeks. It is possible that the diet given during this period was in part responsible and we hope to report more data on this point in the near future. These two dogs we placed on a diet which we have established in other experiments (4) as supplying the minimum of hemoglobin-forming elements, yet being adequate to maintain body weight and health.

Control muscle samples were removed just before the anemia period

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using a technique shown to give samples practically free from blood hemoglobin. The technical procedure is as follows: under ether anesthesia with aseptic precautions the flexor fascia of the fore leg is exposed and split. A portion of the flexor group is raised free by a blunt instrument with no escape of blood. The anterior tendons are cut and finally the upper end of the muscle group is cut across by a single sweep and the muscle sample

TABLE 21

Influence of anemia on muscle hemoglobin; control muscle samples removed

Muscle hemoglobin in milligrams per 100 grams

	DOG NUMBER	
	M-91	M-90
Control muscle right flexor	900 mgm.	720 mgm.
Anemia period	11 weeks	12 weeks
Diaphragm	468	416
Left ventricle	313	434
Right ventricle	266	234
Right rectus	318	420
Left rectus	391	362
Right pectoral	426	304
Left pectoral	399	318
Right triceps	481	479
Left triceps	486	462
Right flexor	500	590
Left flexor	511	469
Right vastus	485	388
Left vastus	496	383
Right gastrocnemius	480	770
Left gastrocnemius	448	895
Right adductor	501	687
Left adductor	459	685
Right spinae	562	845
Left spinae	567	760
Average legs and back	498	641
Weight in kgm	14.4	6.64
Age	4 yrs.	Adult
	Perfused	Not perfused

Anemia level maintained between 40 per cent and 55 per cent hemoglobin.
Dogs were on a diet of baker's bread, standard anemia bread (4) and milk.

plunged into normal saline and massaged vigorously for 2 to 3 minutes to express all blood from the vessels. The muscle is then dried carefully with a towel and prepared as usual for extraction as described in the preceding paper. The leg incision is closed and heals rapidly. A few days later by repeated bleedings the hemoglobin level is reduced to 40 or 50 per cent hemoglobin and maintained at this level by appropriate bleedings.

A series of experiments shows that this method will give a muscle sample (flexor) which is almost identical with the leg muscle on the other side, bleeding-perfusion carried out in the usual fashion described in the first paper immediately after the operative removal. If the operative sample remains in contact with blood even for a few seconds or if the massage of the muscle sample in normal saline is not vigorous and continued in fresh solutions until not a trace of blood escapes during continual massage, we

TABLE 22

Influence of prolonged anemia on muscle hemoglobin

Muscle hemoglobin in milligrams per 100 grams

	DOG NUMBER			
	20-1	20-104	20-103	Average
Diaphragm	531	608	662	600
Right ventricle	335	361	366	354
Left ventricle	342	466	386	398
Right rectus	445	474	282	300
Left rectus	427	465	292	395
Right pectoral	411	458	358	409
Left pectoral	398	470	344	404
Right triceps	590	463	407	486
Left triceps	532	524	437	498
Right flexor	681	648	681	670
Left flexor	652	640	599	630
Right vastus	528	484	366	459
Left vastus	492	514	375	460
Right gastrocnemius	618	518	346	481
Left gastrocnemius	453	521	381	452
Right adductor	427	475	328	410
Left adductor	452	488	487	476
Right spinae	478	474	324	419
Left spinae	533	480	300	438
Average legs and back	536	519	419	489
Anemia period	18 mos.	6 mos.	14 mos.	
Age	5.5 yrs.	4 yrs.	5 yrs.	
Weight in kgm	18.9	10.4	13.5	

Anemia level maintained between 40 per cent and 50 per cent. Dogs on a variety of standard diets to test hemoglobin regeneration. Dog 20-103 not perfused.

will find the operative sample higher in muscle hemoglobin than the control (see tables 6 and 8).

Dog M-91, table 21, shows average of values for legs and back of 498 mgm. as compared with the control flexor removed before the anemia period of 900 mgm. muscle hemoglobin. This dog was freed from blood by the usual method of bleeding-perfusion and in this experiment we can have no doubt but that the muscle hemoglobin level was greatly reduced from the

high control values. This dog was a vigorous, well-conditioned female hound evidently accustomed to much exercise and probably a hunting dog. The portion of the right flexor group not removed at operation gave a muscle hemoglobin level identical with the average. This muscle functioned normally after operation and the dog used the leg freely.

Dog M-90, table 21, gives less satisfactory data than M-91 but we may conclude that the muscle hemoglobin level was somewhat lowered by the long severe anemia. During a routine anemia bleeding this dog died because of too much blood removed. She was at once dissected and the muscles massaged to free them from as much blood as possible. From other experiments we feel that the values given are at least 100 mgm. too high because of the lack of standard perfusion. Some are much higher than others and the experiment is unsatisfactory but in general supports the clear-cut evidence of the other experiment in this table. This dog was a house poodle and would be expected to have low muscle hemoglobin values—compare 84-M, table 8.

In table 22 are given three experiments to show the effect of very long continued severe anemia upon the muscle hemoglobin level. These dogs were used in the experiments of Robscheit-Robbins and Whipple (4) to study the regeneration of blood hemoglobin due to diet factors. The dogs were in perfect condition at all times, quite active and uniform in body weight. The anemia level was uniformly between 40 and 50 per cent hemoglobin using 13.8 grams hemoglobin as 100 per cent. The anemia periods varied from 6 months to 18 months.

It is obvious from table 22 that severe anemia with good general body condition and no complicating factors will not greatly reduce the level of muscle hemoglobin. These dogs with only one-third their normal blood hemoglobin are able to keep their muscle hemoglobin at 500 mgm. per 100 grams. Similar dogs not anemic would show muscle hemoglobin of 600 to 800 mgm.—certainly not over this level—because it must be remembered that these dogs were raised in our kennels and not accustomed to the vigorous outdoor exercise of the farm or hunting dogs which show the highest normal muscle hemoglobin values.

Table 22 shows normal values for heart muscle hemoglobin but somewhat higher values than normal for the diaphragm which averages 600 mgm. muscle hemoglobin as against the leg and back muscle average of 490. In normal non-anemic dogs the diaphragm would be slightly below the average for back and leg muscles. It is probable that the long continued anemia is responsible for increased respiratory rate, and consequent increased muscular activity which invariably results in health in increased muscle hemoglobin values. These dogs are very noisy during all their waking hours and the muscular efforts of barking are certainly more pronounced in this colony than in the dogs reared and trained in the home, farm or kennels where constant inhibition of barking is the rule.

In contrast to anemia conditions we find that nerve section causes the paralyzed muscles to show a rapid decrease in muscle hemoglobin. One cannot say whether this change is due in part to lack of muscular exercise or to lack of muscular tonus or to some trophic factors which are such

TABLE 23

Influence of paralysis on muscle hemoglobin; sciatic nerve section

Muscle hemoglobin in milligrams per 100 grams

	DOG NUMBER			
	24–103	24–99	25–1	Average
Diaphragm	592	670	811	
Right ventricle	352	298		
Left ventricle	373	264	589	
Right rectus	485	511	594	
Left rectus	498	635	687	
Right pectoral	473	501	619	531
Left pectoral	465	447	637	516
Right triceps	527	582	807	638
Left triceps	621	581	950	717
Right flexor	699	705	875	759
Left flexor	693	720	902	772
Right vastus	491	623	727	614
Left vastus	506	588	760	618
Right gastrocnemius	378	545	569	474
Left gastrocnemius	657	608	692	652
Right anterior tibial	363	335		349
Left anterior tibial	675	560		617
Right gracilis	547			
Left gracilis	553			
Right adductor	256	580	614	483
Left adductor	690	591	847	709
Right semi-membranosus	334	470	675	493
Left semi-membranosus	465	581	790	612
Right spinae	686	603	872	720
Left spinae	682	626	764	691
Heart weight in grams	107.5	65.7	111.4	
Weight in kgm.	13.5	8.7	17.7	
Sciatic section	47 days	33 days	27 days	

Dog 24–103, right tibial (operative) 11.1 gm. weight.
left tibial (control) 19.2 gram weight.
Average muscle hemoglobin for paralyzed leg muscles, 455.
Average muscle hemoglobin for control leg muscles, 658.

familiar topics of discussion for physiologists. However, the fact remains that sciatic nerve section will give a definite decrease in muscle hemoglobin which becomes more striking from week to week (table 23). Our periods of observation vary from 27 days to 47 days following nerve section. After

several weeks the muscles show atrophy and gross differences in color. In some muscles the decrease is almost to 50 per cent of the control leg.

The experimental procedure is very simple. Under ether anesthesia the sciatic nerve of the right leg is exposed close to the great trochanter and sectioned. The wound is closed and the dog is very little disturbed. He can walk with only slight difficulty and use the operated leg well except for the fact that the paw turns under in walking. The usual bleeding-perfusion technique was used to free the muscles from circulating hemoglobin.

Discussion. One thing which comes out pretty clearly in table 22 is the inability of the anemia demand to rob the muscles of their store of hemoglobin. In these anemia dogs there is a maximal demand for circulating hemoglobin and this demand continues for months at maximal intensity. The bone marrow shows extreme hyperplasia and the body uses everything available in the diet and host cell katabolism for this emergency formation of circulating hemoglobin. Yet in this great emergency the muscle hemoglobin level is very slowly and to a limited degree disturbed provided the dog is active and healthy. The demands of the active muscle for its hemoglobin evidently are honored as meticulously in the internal metabolism clearing house as are the demands of the blood forming organs. This does away at once with the notion that there is any parallelism between the blood hemoglobin level and the muscle hemoglobin level, as one might suspect from the curve of normal increase in growing pups. It is a fortunate adjustment that these two levels do not go in parallel as a fatal outcome would result if the heart and skeletal muscle hemoglobin levels fell promptly with the blood hemoglobin level—in such circumstances any severe anemia would cause death due to muscular and cardiac weakness.

SUMMARY

Prolonged severe anemia may reduce the level of muscle hemoglobin slowly. If the original level was high the reduction may even amount to 30 or 40 per cent. If the dogs are healthy and active this reduction will scarcely go below 400 to 500 mgm. muscle hemoglobin per 100 grams. We may see even *lower normal* values in quiet house dogs. We believe that exercise is more important than anemia in determining the muscle hemoglobin level in the dog.

Anemia demands cannot rob the striated muscle of its hemoglobin. The body ranks the necessity for muscle hemoglobin maintenance on a par with the need for blood hemoglobin production and from the standpoint of survival of the individual this is a fortunate circumstance. It also shows the importance of muscle hemoglobin in body economy and general pigment metabolism.

Muscular paralysis (sciatic nerve section) is followed by a fairly rapid loss of muscle hemoglobin becoming more noticeable from week to week. After a period of 7 weeks some muscles may contain only one half the muscle hemoglobin found on the control normal side.

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PHYSIOLOGICAL REVIEWS

PURPOSES

The main purpose of PHYSIOLOGICAL REVIEWS is to furnish a means whereby those interested in the physiological sciences may keep in touch with contemporary research. The literature, as every worker knows, is so extensive and scattered that even the specialist may fail to maintain contact with the advance along different lines of his subject. The obvious method of meeting such a situation is to provide articles from time to time in which the more recent literature is compared and summarized. The abstract journals render valuable assistance by condensing and classifying the literature of individual papers, but their function does not extend to a comparative analysis of results and methods. Publications such as the Ergebnisse der Physiologie, the Harvey Lectures, etc., that attempt this latter task, have been so helpful as to encourage the belief that a further enlargement of such agencies will be welcomed by all workers. It is proposed, therefore, to establish a journal in which there will be published a series of short but comprehensive articles dealing with the recent literature in Physiology, using this term in a broad sense to include Bio-chemistry, Bio-physics, Experimental Pharmacology and Experimental Pathology.

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